Jari Olavi Summanen

A Chemical and Ethnopharmalogical Study on *Phyllanthus emblica* (Euphorbiaceae)

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Science of the University of Helsinki, for public criticism in Auditorium XII, on August 4th,1999, at 12 o'clock noon.

University of Helsinki Department of Pharmacy
Division of Pharmacognosy

Supervisors: Prof. Raimo Hiltunen, Ph.D.
Division of Pharmacognosy
Department of Pharmacy
University of Helsinki
Finland

Prof. Heikki Vuorela, Ph.D.
Division of Pharmacognosy
Department of Pharmacy
University of Helsinki
Finland

Reviewers: Docent Ilkka Ojanperä, Ph.D.
Department of Forensic Medicine
University of Helsinki
Finland

Prof. Raimo K. Tuominen, M.D.
Division of Pharmacology and Toxicology
Department of Pharmacy
University of Helsinki
Finland

Opponent: Prof. Adolf Nahrstedt, Dr. rer. nat.
Institut für Pharmazeutische Biologie
und Phytochemie der Universität
Hittorstraße 56
D-48149 Münster
Germany

ISBN 951-45-8677-8

Helsingin yliopiston verkkojulkaisut, 1999

Contents

Preface

This work was carried out at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki during the years 1994–1998.

I wish to express my gratitude to Professor Raimo Hiltunen, Head of the Pharmacognosy Division and Head of the Department of Pharmacy, for his support during the course of this study and for placing excellent facilities for my work.

My sincere gratitude is due to my supervisor, Professor Heikki Vuorela, whose encouraging guidance and stimulating discussions have led my work. Apart from scientific stuff, thanks are also due to openmindedness to discuss anything anytime during all these years.

My special thanks go to Professor, M.D. Heikki Vapaatalo, Department of Pharmacology and Toxicology, Institute of Biomedicine, University of Helsinki for introducing me the subject together with Assoc. Prof., Ph.D. M. Zaini Asmawi, Universiti Sains Malaysia, Penang, Malaysia. Special thanks are also due to Docent, M.D. Hannu Kankaanranta, Docent, M.D. Eeva Moilanen and Docent, Ph.D. (Pharm.) Pia Vuorela. Without their valuable collaboration my work would never have been started.

I want to express my appreciation to Dr. Michael Pasternack for the patch clamp experiments and Prof. Dr. Kid Törnquist, who introduced me to calcium antagonistic test methods. I would also like to thank them for the encouraging discussions and advice throughout the course of the experiments and writing.

Mr. Teijo Yrjönen, M.Sc. (Pharm.) deserve special thanks for valuable cooperation during the course of this study. My thanks are also due to co-authors, Prof. Marja-Liisa Riekkola, Docent Ilkka Ojanperä, Docent Heikki Haario, Docent Heli Sirén, M.Sc. (Pharm.) Arja Ihantola-Vormisto, M.Sc. (Pharm.) Krista Marjamäki, M.Sc. (Chem.) Anna-Kaisa Pelander, M.Sc. (Pharm.) Jussi-Pekka Rauha, and M.Sc. (Pharm.) Päivi Tammela.

I'm very grateful to Docent, Ph.D. Ilkka Ojanperä and Professor, M.D. Raimo K. Tuominen for their careful reviewing of the manuscript and most valuable comments.

I am especially grateful to pharmaceutical company Oy Nycomed Ab, a subsidiary of Nycomed Amersham plc, for keeping me as an employee during these years. Special thanks for understanding my peculiar working hours during my studies are due to Managers Mrs. Auni Saarinen and Mr. Raimo Skottman.

Warm thanks are due to John Derome, For.Lic., for revising of the language in the publications as well as the thesis.

Sincere thanks are due to my colleagues at the Division of Pharmacognosy, who have made the period when I worked on the thesis very inspiring. I also owe thanks to Professor Lars Bohlin and all his staff members at the Division of Pharmacognosy during my visits at the University of Uppsala, Sweden.

Many thanks are due to Librarian Seija Tikkanen for the valuable library service. My warm thanks go also to Engineer Sandy Fuchs, for his reliable help with chromatographic media and instruments. Laboratory engineer M.Sc. (Phys.) Pentti Peltola deserves warm thanks for technical assistance and for lending necessary tools, *e.g*. gas mask due to solvent leak in the OPLC equipment.

Thanks are also due to all my relatives, friends and colleagues outside and in Finland. My warm thanks go to the pharmacy students organisation Yliopiston farmasiakunta — Universitetets farmaciekår ry. (commonly known as YFK) officials and members. It’s been a great honour to hold the curators confidential post since 1995.

My best and warmest thanks go to my family, Oona, Kaisa and Eija for their support and understanding during the course of this work. Special thanks go also to my parents for keeping my jack-of-all-trades skills updated with several projects in countryside and out at sea.

This study was partially financed by the Research and Science Foundation of Farmos, Finnish Cultural Foundation/Elli Turunen Foundation, Association of Finnish Pharmacies, Emil Aaltonen Foundation, the Society of Pharmaceutical Sciences in Finland, and the University of Helsinki, who are gratefully acknowledged.

Helsinki, June 1999

Abstract

The use of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) in the separation of phenolic antioxidants was investigated. A simple and fast MEKC method provided sufficient selectivity for the satisfactory resolution of gallates, butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT). Application of the marker technique improved the repeatability of the analysis and the reliability of identification. The standard deviations for the migration indices were less than 1 % and the technique can therefore be used for rapid purity testing of real samples extracted from plant material, *i.e. Phyllanthus emblica* L. (Euphorbiaceae) leaf extracts.

The Automated Multiple Development (AMD) technique is suitable for the separation of multicomponent mixtures in thin layer chromatography (TLC). The main challenge in optimization AMD is the handling of the numerous instrumental parameters. The wide variation in the polarity of phenolic compounds, *e.g.* in medicinal plants, can cause problems in identification and separation. Systematic experiments were therefore performed to evaluate gradient elution in AMD for finding a suitable set of operating parameters for the separation of a phenolic reference mixture. Careful optimization leads to an efficient and reliable separation that can be repeated automatically. AMD TLC is a useful tool that provides more powerful screening than conventional, non-instrumental TLC methods.

The effect of experimental parameters on the separation of phenolic coumpounds was studied by densitometry and video TLC-documentation system. Both the video and densitometer methods are suitable for any analyst lacking the special skills needed to make documents on TLC developments. The video storage system is easy to use when most of the parameters are kept constant. The strong points of the video documentation are the independence of the mode of separation and the structure of the chromatoplate used, the speed of evaluation, and the archiving the captured image for further reporting purposes, *e.g.* to satisfy the demands of Good Laboratory Practise (GLP). The limitation of both reflectance densitometry and video documentation is the detection of compounds distributed vertically inside the depth of the chromatoplate.

A computer program for the mobile phase optimization of TLC was employed to enhance the quality of TLC separations. The desirability function technique was combined with the "PRISMA" model. The study showed that the dependence between the eluent composition and retardation for the phenolic test mixtures can be expressed to a high degree of accuracy using quadratic regression models. The optimum eluent mixtures for the separation of compounds were read from the contour plot inside the horizontal plane of the "PRISMA", and a good separation was achieved using the optimized solvent combination.

P. emblica L. (Euphorbiaceae), a tree growing in subtropical and tropical areas of Far-Eastern countries has been reported to contain constituents with variable biological effects. The activities of crude leaf extracts were evaluated in human polymorphonuclear leukosytes (PMNs) and platelets. The study showed that the plant leaves have antineutrophil and antiplatelet properties *in vitro*. This agrees

with the anti-inflammatory and antipyretic usage of this tree in traditional medicine by rural populations in Asia.

Calcium (Ca^{2+}) is a key mediator of various intracellular processes. Excitable cells contain voltage dependant, receptor operated and stretch operated channels at the plasmalemma. These channels enable the cells to increase cytosolic Ca^{2+} levels. Calcium channels are highly interesting because they are targets for the drugs used in cardiovascular therapy. The cells of rat pituitary gland (GH_4C_1) have been found to possess voltage operated Ca^{2+} channels (VOCCs), and can therefore be used in models studying compounds that interact with Ca^{2+} channels. The calcium transport activity of 9 phenylpropanes and -metanes, and 20 flavonoids was studied in cultured rat pituitary cells (GH_4C_1) in order to determine their possible interaction with VOCCs. Flavones (flavone and isoflavone genistein) and phenylmetane derivative octyl gallate displayed clear inhibition of Ca^{2+} entry. The action of the octyl gallate and quercetin on VOCCs was further studied by the means of whole-cell patch-clamp technique. Quercetin markedly enhanced both transient and delayed Ca^{2+} currents, indicating that quercetin may affect both T- and L-type VOCCs. Onset of action of octyl gallate was clearly slower than that of quercetin.

List of Original Publications

I Summanen J., Vuorela H., Hiltunen R., Sirén H., Riekkola M.-L.: Determination of phenolic antioxidants by capillary electrophoresis with ultraviolet detection. *J. Chromatogr. Sci.* 1995; 33 (12): 704–711.

II Summanen J., Hiltunen R., Vuorela H.: The choice of parameters in the optimization of Automated Multiple Development. *J. Planar Chromatogr.* 1998 (11):16–24.

III Summanen J., Yrjönen T., Hiltunen R., Vuorela H.: Influence of densitometer and video documentation settings in the detection of plant phenolics by TLC.*J. Planar Chromatogr.* 1998 (11): 421–427.

IV Pelander A., Summanen J., Yrjönen T., Haario H., Ojanperä I., Vuorela H.: Optimization of separation in TLC using desirability functions and mixture designs according to "PRISMA". *J. Planar Chromatogr.* 1999 (Manuscript No. SN-925, accepted for publication).

V Ihantola-Vormisto A., Summanen J., Kankaanranta H., Vuorela H., Asmawi M.Z., Moilanen E.: Anti-inflammatory activity of extracts from leaves of *Phyllanthus emblica*. *Planta Med.* 1997 (63): 518-524.

VI Summanen J., Vuorela P., Rauha J-P., Tammela P., Marjamäki K., Pasternack M., Törnqvist K., Vuorela H.: Contrasting effects of simple aromatic compounds and flavonoids on calcium fluxes in clonal rat pituitary GH_4C_1 cells.*J. Pharmacol. Exp. Ther.* 1999 (submitted for publication).

These publications will be referred to in the text by their Roman numerals. In addition, some unpublished results are presented.

Reprints are not included in this PDF version.

List of Abbreviations

AMD	automated multiple development
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CCD	charge coupled device
CCA	calcium channel antagonist(s)
CE	high performance capillary electrophoresis
CMC	critical micelle concentration
COX	cyclooxygenase
CZE	capillary zone electrophoresis
CV	coefficient of variation
D	desirability value
D_0	overall desirability
$D(R_S)$	desirability function for the resolution
DTL	details (regulates the sharpness of video images)
E_{eff}	effective electric field strength
FF	forced flow
FMLP	*N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine
GC	gas chromatography
GH_4C_1 cells	cultivated cells from rat pituitary gland
HPLC	high pressure liquid chromatography
HPTLC	high performance thin layer chromatography
IC_{50}	concentration yielding 50 % inhibition
IR	infrared
k_P	capacity factor for planar chromatography
LC	liquid chromatography
LTB_4	leukotriene B_4
MEKC	micellar electrokinetic capillary chromatography (= MECC)
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
NP	normal phase, silica
NP-TLC	normal phase thin layer chromatography
OPLC	overpressured layer chromatography
PAF	platelet activating factor
PMN	polymorphonuclear leukocytes
P_S	selectivity point
R^2	coefficient of determination
R_f	retardation factor
RP	reversed phase
RP-TLC	reversed phase thin layer chromatography
R_S	resolution
RSD	relative standard deviation
SD	standard deviation

SDS	sodium dodecyl (=lauryl) sulfate
S_T	solvent strength
TLC	thin layer chromatography
TXB_2	thromboxane B_2
UV	ultraviolet
UV/VIS	ultraviolet / visible
ν	migration velocity
VIS	visible
VOCC(s)	voltage operated calcium channel(s)
ν_{EO}	electro-osmotic velocity
w_h	peak width at half height

1. Introduction

Molecular biology, genetic engineering and computational chemistry have created considerable potential within the pharmaceutical industry without the need to explore nature's chemical diversity. In the synthetic drug development of compounds, either a search is made through the inventory of substances earlier synthesized to find relatives to the theoretical molecule, or the theoretical molecules and analogues are synthesized. In spite of this, organic chemists have realised that plant species contain a bewildering diversity of secondary metabolites. In indigenous cultures higher plants have formed the basis for the treatment of diseases since the earliest times. Accuracy in recording or observing the medical use of a plant, determining whether the ethnomedical use can be demonstrated under scientific conditions in the laboratory, chemical characterization of the compound(s), and the role of the placebo effect, are important issues that need to be verified in the development of drugs of plant origin.

During the last two decades there has been an upsurge in the search for new plant-derived drugs containing medicinally useful alkaloids, glycosides, polyphenolics, steroids, and terpenoid derivatives. FARNSWORTH et al. (1985) identified 119 secondary metabolites, isolated from higher plants, that were being used globally as drugs. It has been estimated that 80 % of the world's population still use traditional medicine for their primary health-care needs (FARNSWORTH 1988). Secondary metabolites isolated from medicinal plants can serve as precursors or models for the preparation of effective agents through semi-synthesis or lead-based total synthesis. Many important modern plant drugs, such as vinblastine and vincristine, have been discovered by following leads from traditional medicines (CARTER et al. 1976). Other drugs have been produced chemically, using the natural product as a template, *e.g.* butylmorphine and sodium chromoglycate. Traditional medicine has thus formed a basis for the creation of an interdisciplinary science, 'ethnopharmacology' Ethnopharmacology has been defined as the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man (BRUHN et al. 1981). In accordance with the objectives of ethnopharmacology, new antifungal compounds have recently been found (BIERER et al. 1995).

A number of chromatographic techniques have been developed into important tools in the field of analytical and preparative natural product chemistry associated with the characterization of biologically active compounds (HÄRMÄLÄ 1991, LIANG 1997, NOREEN 1997). High performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have been the conventional methods used in the analysis of secondary metabolites originating from plants. The use of computer assisted expert systems and automated development techniques in the search for analytical conditions in HPLC and TLC, have recently become important tools in determining and optimizing the analytical conditions for multicomponent mixtures (OUTINEN et al. 1998, PELANDER et al. 1999). Reliable and fast camera-, video- and laser-recording techniques have challenged densitometers in the documentation of TLC analysis, and in producing chromatograms for large-scale screening, or when Good Laboratory Practice (GLP) has to be followed (VÉGH et al. 1998,

VOVK et al. 1998, PETROVIC et al. 1999). Capillary electrophoresis (CE) has been developed for the separation of a broad range of analytes. Due to the number of general advantages, *e.g.* shorter separation times, less reagent consumption, cheaper columns, and higher resolution, more attention has been paid to the potential of CE for the analysis of plant- derived compounds, and especially in the systematic validation and characterization of real medicinal plant samples (TOMÁS-BARBERÁN 1995, LIANG 1997).

The number of *in vitro* and *in vivo* assays available for biological activity is large (VOGEL and VOGEL 1997). With the help of high throughput screening (HTS) and synthetic support from medicinal chemistry, two new natural antifungal products have been discovered (BIERER et al. 1995). Although on-line techniques for the investigation of biological activity are available, new therapeutic uses of known chemical compounds can be as important for health care as identifying novel structures with known activities (CORDELL 1995, PEZZUTO 1997). Current medicinal knowledge of the activity of plant phenolics indicates that useful drugs may be developed from them in the future, or that they could be used as templates for further research and development (VUORELA et al. 1997, KAPIOTIS et al. 1997).

2. Aims of the Study

This study was focused on the analysis of phenolic compounds and the screening of their biological activity using *Phyllanthus emblica* L. (Euphorbiaceae) as the source of biological material. The usefulness of modern, electron-driven separation methods and thin layer chromatographic methods were investigated in the separation of phenolic compounds. The anti-inflammatory activity of a number of *P. emblica* L. leaf extracts was measured in order to verify the traditional uses of this ethnomedical plant. Simple aromatic compounds and flavonoids were screened for possible calcium antagonistic activity by measuring calcium uptake in rat pituitary GH_4C_1 cells. The results were further confirmed using the whole-cell patch-clamp technique.

The specific aims of this study were:

- to evaluate the use of high-performance capillary electrophoresis (CE) as an analytical technique for phenolic compounds and as a rapid method for screening of phenolic compounds from plant extracts (**I**)
- to investigate the use of modern TLC methods in the separation of plant phenolics by studying the effect of chromatographic parameters in Automated Multiple Development (AMD) (**II**), to evaluate the video documentation system as an alternative detection method to the densitometer scanning technique (**III**), and to study the applicability of computer-assisted optimization programs in TLC separations (**IV**)
- to study the anti-inflammatory activity of *P. emblica* L. leaves (**V**), and to screen simple aromatic compounds and flavonoids with respect to their possible calcium antagonistic activity (**VI**).

3. Review of the Literature

3.1. *Phyllanthus*

3.1.1. Botanical aspects

The plant genus *Phyllanthus* (Euphorbiaceae) is widely distributed in most tropical and subtrobical countries. It is a very large genus consisting of approximately 550 to 750 species and is subdivided into 10 or 11 subgenera: *Botryanthus*, *Cicca*, *Conani*, *Emblica*, *Ericocus*, *Gomphidium*, *Isocladus*, *Kirganelia*, *Phyllanthodendron*, *Phyllanthus*, and *Xylophylla* (UNANDER et al. 1995, CALIXTO et al. 1998). *Phyllanthus emblica* L. is a tree of small or moderate size with a greenish-grey bark and greenish-yellow flowers, formed in axillary clusters. The feathery leaves are linear-oblong, with a rounded base and obtuse or acute apex. The tender fruits are green, fleshy, globose and shining, and change to light yellow or brick-red when mature. It grows in tropical and subtropical parts of China, India, Indonesia, and on the Malay Peninsula. The Malaysian variety has more scurfy branchlets and the immature fruit is top-shaped. The name of the Malacca river and town is believed to have been derived from the name of this tree. The origin of the name is from Sanskrit (Melaka, Malaka). In Tamil the tree is known as Nelli, the fruit Nellikai and in Bangladesh Amlaki, Amla in Hindi, and Yeowkan in Chinese. The fruits are known as Amalakam and Sriphalam in Sanskrit, Emblic myrobalam and Indian gooseberry in English, and Phylontha emblic in French.

3.1.2. Phytochemistry

A wide range of plant species belonging to the genus *Phyllanthus* have been phytochemically investigated. Among the studied species, *P. niruri*, *P. urinaria*, *P. emblica*, *P. flexuosus*, *P. amarus*, and *P. sellowianus* have received the most phytochemical and biological attention. According to the literature, research has either been focused on isolating all the substances in these plants, or on determining a specific class of natural products (CALIXTO et al. 1998). The *P. emblica* L. tree contains the different classes of constituents listed in **Table 1** and references there in.

Fig. 1. *Leaves of* Phyllanthus emblica *L. (Euphorbiaceae).*

The complexity of the mixture of compounds and the presence of several compounds in small concentrations can make the isolation and identification of the substances present in this genus very laborious. Different environmental conditions can also affect the chemical constitution of the plants, and differing interpretation of the spectral data of the complex structures has been reported to result in considerable confusion (JUNIOR and VIZZOTTO 1996). The choice of solvent in the isolation of compounds has proved to be crucial, because the use of ethanol or methanol may lead to the production of artefacts, *e.g.* ethyl gallates or methyl gallates, during the extraction process (CALIXTO et al. 1998).

Table 1. *The classes of chemical constituents reported in* Phyllantus emblica *L. (Euphorbiaceae) in the literature.*

Class	Compound	Occurence	Reference
Alkaloid	phyllantine phyllantidine zeatin zeatin nucleotide zeatin riboside	leaves, fruit, and tissue cultures leaves fruit	KHANNA and BANSAL 1975 RAM and RAO 1976
Benzenoid	chebulic acid chebulinic acid chebulagic acid	leaves	THERESA et al. 1965, 1967
	gallic acid		THERESA et al. 1965, 1967 BASA and SRINIVASULU 1987
	ellagic acid	leaves	THERESA et al. 1965, HUI and SUNG 1968, SUBRAMANIAN et al. 1971
		fruit	DESAI et al. 1977
	amlaic acid		THERESA et al. 1967
	corilagin 3-6-di-O-galloyl-glucose ethyl gallate	fruit	SRIVASTAVA and RANJAN 1967
	ß-glucogallin	leaves fruit	THERESA et al. 1967 SRIVASTAVA and RANJAN 1967
	1,6-di-O-galloyl-ß-D-glucose 1-di-O-galloyl-ß-D-glucose putranjivain A digallic acid	fruit	EL-MEKKAWY et al. 1995
	phyllemblic acid emblicol	fruit	PILLAY and IYER 1958
	music (=galactaric) acid		BASA and SRINIVASULU 1987
Furanolactone	ascorbic acid	fruit	DAMORADAN and SRINIVASAN 1935, QUADRY et al. 1962, SHAH and HAMID 1968
		leaves	BASA and SRINIVASULU 1987

Class	Compound	Occurence	Reference
Diterpene	gibberellin A-1 gibberellin A-3 gibberellin A-4 gibberellin A-7 gibberellin A-9		RAM and RAJA 1978
Triterpene	lupeol	fruit leaves	DESAI et al. 1977 HUI and SUNG 1968
Flavonoid	leucodelphinidin	leaves	LAUMAS and SESHARDI 1958
	kaempherol	leaves	SUBRAMANIAN et al. 1971
	kaempherol-3-glucoside	leaves	
	rutin	leaves	YRJÖNEN et al., unpublished results
	quercetin	leaves	YRJÖNEN et al., unpublished results
	kaempherol-3-O-ß-D-glucoside quercetin-3-O-ß-D-glucoside	fruit	EL-MEKKAWY et al. 1995
Sterol	ß-sitosterol	leaves	HUI and SUNG 1968
Carbohydrate	Acidic and neutral polysaccharides fruit	NIZZAMUDDIN et al. 1982	
	Glucose	leaves	THERESA et al. 1967

3.1.3. Ethnopharmacology

Phyllanthus species have long been used in folk medicine to treat a broad spectrum of disorders, and there are numerous references to controlled assays. UNANDER et al. (1990, 1991, 1992, 1995) published an extensive, four-part survey of the usage of bioassays in the genus *Phyllanthus*. These articles cover published data concerning traditional uses, as well as the results of laboratory assays. Recently, seven ellagitannins from *P. myrtifolius* and *P. urinaria* were reported to show activity against Epstein-Barr virus DNA polymerase (EBV-DP) (LIU et al. 1999). However, the effects against chronic infection with hepatitis B virus (HBV) or related viruses remain negative.

P. emblica L. has been used for anti-inflammatory and antipyretic treatments by rural populations in its growing areas. Malays use a decoction of its leaves to treat fever (BURKILL 1966). In Indonesia, the pulp of the fruit is smeared on the head to dispel headache and dizziness caused by excessive heat (PERRY 1980). The earlier chemical findings and biological activities have since been confirmed with more advanced techniques. Active principles or extracts of *P. emblica* L. have been shown to possess several pharmacological actions, *e.g.* analgesic, anti-inflammatory, antioxidant, chemoprotective, hypolipidaemic and anti-HIV-1 (Human immunodeficiency virus-1) activities (**Table 2**).

***Table 2**. Recently reported biological effects of* Phyllanthus emblica *L. (Euphorbiaceae).*

Biological effect	**References**
Antagonistic activity against genotoxic chemicals, anticlastogenicity*in vitro*	GIRI and BANERJEE 1986, DHIR et al. 1990 and 1991, ROY et al. 1992, NANDI et al 1997
Antimicrobial activity, *in vitro*	SANKARANARAYANAN and JOLLY 1993, YRJÖNEN et al., unpublished results
Antioxidant activity *in vitro*	JOSE and KUTTAN 1995, GHOSAL et al.1996, KUMAR and MÜLLER 1999
Anti-inflammatory activity, *in vivo and in vivo*	ASMAWI et al. 1992, JANTAN et al. 1996 IHANTOLA-VORMISTO et al. 1997
Hepatoprotective activity, prevention of hepatocarcinogenesis, *in vitro a*nd *in vivo*	ROY et al. 1991, GULATI et al. 1995, JOSE et al. 1997, 1999
Hypolipidaemic, *in vivo and in vitro*	THAKUR 1985, MAND et al. 1991, JACOB et al. 1988, MATHUR et al. 1996
Enhancer of natural killer cell (NK) activity, *in vitro*	SURESH and VASUDEVAN 1994
Inhibition of human immunodeficiency virus-1 (HIV-1) reverse transcriptase, *in vitro*	EL-MEKKAWY et al. 1995 KUSUMOTO et al. 1995
Prevention of experimental acute pancreatitis, *in vivo*	THORAT et al. 1995
Protection against radiation-induced chromosome damage, *in vitro*	YADAV 1987

Although research has been carried out on the chemical components in the genus *Phyllanthus*, their pharmacological properties are generally not well known. Nevertheless, the review by CALIXTO et al. (1998), support the experience of traditional medicine that *P. emblica* and other plants belonging to this genus might have beneficial therapeutic actions in the management of certain disturbances such as inflammatory reactions, intestinal problems, hepatitis B, kidney and urinary problems.

3.1.4. Importance of aromatic plant phenols in biological systems

Plants contain a variety of secondary metabolites that play an important role in plant physiology and in the interaction between the plant and its environment. Several thousand phenolic compounds occur widely throughout the plant kingdom (HARBORNE 1995, HASLAM 1998). A large number of the phenols which possess distinct biological activities, *e.g.* simple benzenoids and flavonoids, are biosynthesized via shikimic acid and acylpolymalonate pathway.

Natural phenolic acids belong to two different classes, hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA). They are derived from two nonphenolic molecules, benzoic and cinnamic acids, respectively. Phenolic acids are widely represented in fruits, although their distribution may vary considerably according to species, cultivar, and physiological stage. Phenolic acids are of great interest to man because they contribute to the sensory and nutritional qualities. Most natural antioxidants possess a polyphenolic structure, and a large number of reviews have been published concerning their origin and role (HO 1992a, SHAHIDI et al. 1992, HARBORNE 1994, MELTZER and MALTERUD 1997, HASLAM 1998). Phenolic acids can act as endogenous precursors for many of the other phenolic molecules found in plants. Flavonoids may contain partial structures derived from either shikimic acid or acylpolymalonate routes. Over 4000 different flavonoids have been described, and the number is still increasing (MIDDLETON and KANDASWAMI 1994, HASLAM 1998). The classes of flavonoids vary in type and quantity due to variations in plant growth, environmental conditions and maturity. Subgroups of flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids, share the common skeleton of diphenylpyrans (C6-C3-C6), *i.e.* two benzene rings (A and B) are linked through a heterocyclic pyran or pyrone ring (C) in the middle.

Natural phenolic compounds make a considerable contribution to the nutritional quality of fruits and fruit products, which play an important role in the daily diet. They also play a key role in antioxidative defence mechanisms in biological systems and they may have inhibitory effects on mutagenesis and carcinogenesis. Attention has turned to plant phenols because the use of synthetic antioxidants has been falling off due to their suspected action as cancer promotors (HO 1992a). Caffeic acid, gallic acid and gallic acid derivatives (methyl-, lauryl and propylgallates) show strong antioxidant properties and act as free radical acceptors (HO 1992b). They are widely used as food additives to protect lipid structures. Nevertheless, phenols can simultaneously have pro-oxidant effects, *i.e.* cause tissue damage by producing reactive oxygen species (ROS), and their consumption should be regarded with caution (ARUOMA et al. 1993). The important biological activities of simple benzenoids, *e.g.* chlorogenic, caffeic, ferulic, gallic and ellagic acids, are probably due to their cytoprotective activity and possible inhibitory effects on carsinogenesis, mutagenesis and tumorigenesis (LESCA 1983, STICH and ROSIN 1984, CHANG et al. 1985, MUKHTAR et al. 1988, MACHEIX and FLEURIET 1998, VIEIRA et al. 1998, HASLAM 1998, KUMAR and MÜLLER 1999). Flavonoids have a range of biological effects in a great number mammalian cell systems, *in vitro* as well as *in vivo*. Flavonoids have been shown to possess antiviral and endocrine effects, activity on mammalian enzymes, effects on the modulation of immune and inflammatory cell functions, effects on smooth muscles, and effects on lipid peroxidation and oxyradical production (HARBORNE 1994, FORMICA and REGELSON 1995, MELTZER and MALTERUD 1997). Since flavonoids are regular constituents of our every day diet, their possible genotoxic, carcinogenicity, and mutagenicity related properties have recently received increasing attention (RICE-EVANS and PACKER 1998). Although evidence from human and animal, as well as *in vitro* experiments, support the hypothesis that flavonoids promote health, it is possible that interactions with other dietary constituents or lifestyles may override any subtle positive effects of flavonoids in humans (SAMMAN et al. 1998). Genetic engineering might lead to the production of fruits in which the phenolic metabolism is over- or underexpressed (DIXON and PAIVA 1995). The de-

velopment of plants depends on whether better nutritional quality or the accumulation of antioxidative properties and interactions with human health are preferred.

3.1.5. Analysis of phenolic compounds

The chosen method usually involves some form of chromatography in which plant material or products containing phenolic compounds must be analyzed, isolated, or quantitatively assayed. Quantitative analysis is usually carried out by high performance liquid chromatography (HPLC), thin layer chromatography (TLC) or high performance capillary electrophoresis (CE) (IBRAHIM and BARRON 1989, TOMAS-BÀRBERAN 1995, MARKHAM and BLOOR 1998).

3.2. Separation Methods

Column and planar liquid chromatographic techniques have always been developed in constant mutual interaction. The intensive development of high performance liquid chromatography (HPLC) has also resulted in the synchronous development of planar chromatography in the form of sophisticated instrumental techniques. In this study, two chromatographic techniques were used in the analysis of simple phenolic compounds and flavonoids. The principles of an electron-driven separation method, high performance capillary electrophoresis (CE), and thin layer chromatography (TLC) are described in the following.

3.2.1. Planar chromatography

Planar chromatography includes all those chromatographic techniques performed in a planar field of variable thickness and surface. It has several advantages over column chromatographic separations, *e.g.* simultaneous separation of several samples, two-dimensional development, detection by specific/sensitive colour reagents, specific contact detection (*e.g.* X-ray film), visual detection of ultraviolet (UV-) absorbing compounds, and the production of a chromatogram using a developing solvent (STAHL 1967, BEESLEY 1985, GEISS 1987, TOUCHSTONE 1990, KALÀSZ and BÀTHORI 1997).

Thin layer chromatography (TLC)

TLC or high performance TLC (HPTLC) is primarily used as an inexpensive method for separation, for qualitative identification, or for the semiquantitative visual analysis of samples. TLC is thus often described as a pilot method for HPLC (ROZYLO and JANICKA 1991, 1996). However, recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis (WEINS and HAUCK 1996, KALÀSZ and BÀTHORI 1997). The use of TLC/HPTLC have expanded considerably due to the development of forced flow

(FF) and gradient TLC methods, stationary and mobile phase selection, as well as new quantitation methods (POOLE and POOLE 1994, SHERMA 1994).

Instrumental development techniques in TLC

New separation and detection techniques, as well as coupling with spectroscopic methods, has decreased the need for manual operation and eliminated important sources of error. The accuracy, reproducibility and specificity of the TLC/HPTLC methods have therefore been increased. Various application devices have markedly increased the precision and reliability of sample application that is very crucial step in quantitation (JAENCHEN 1996a, 1996b). A forced-solvent flow can be used in overpressured layer chromatography (OPLC), rotation planar chromatography (RPC), and high speed (HS)TLC with electroosmosis. Independent optimization of the mobile phase velocity, higher efficiency, lower separation times, use of solvent gradients and solvents that do not wet the layer are the main advantages of FF-TLC (SHERMA 1994). Separation can be performed off-line, *i.e.* started with a dry layer, but totally on-line OPLC can also be used (MINCSOVICS and TYIHÁK 1988). Automated developing instruments have considerably decreased the error caused by the use of simple developing chambers. Their multistep development can markedly enhance TLC separation (MATYSIK 1996, MATYSIK and GIRYN 1996). In automated multiple development (AMD), the plate is automatically developed through and by preset mobile phases, and subsequent drying is also carried out (BURGER and TENGLER 1986, GRINBERG 1990, EBEL and VÖLKL 1990). The AMD technique has been applied in various multicomponent analyses during the past ten years (EBEL et al. 1987, MENZIANI et al. 1990, LODI et al. 1991, QUECKENBERG et al. 1993, POOLE et al. 1995, GOCAN et al. 1996) and also a self-constructed automated system has recently been introduced by OMORI et al. 1998. In spite of the advantages, the efficacy of gradient multistep elution will never be higher than that of continous gradient elution in LC.

Detection techniques in TLC

Detection in TLC can be based on physical (e.g. UV/VIS, IR) or microchemical (postderivatization with general or specific reagents) methods (JORK et al. 1990). Screening of plant extracts on TLC can also be based on biological activity, *e.g.* antimicrobial activity, and not only on the chemical structures (HOSTETTMANN et al. 1997). Quantitative detection can be carried out *in situ* , or the analytes can be extracted from the layer and analyzed using another detection method (TOUCHSTONE 1992, CSERHÁTI and FORGÁCS 1997, 1998).

In densitometric detection, the UV/VIS absorbance, fluorescence or quenched fluorescence is measured directly from the plate in the transmittance or reflectance mode (TOUCHSTONE 1992). At present, most of the quantitative TLC analyses are performed by an optical slit scanning or point scanning densitometer that measures absorbance or fluorescence *in situ* (POLLAK 1987, SHERMA 1994). A number of different TLC scanners, combined with sophisticated software have revolutioned quantitative determination in TLC (EBEL 1996). One trend during the past two years has been the use of image analysers, which might not equal the specificity or accuracy of densitometric evaluation, but may be adequate in many analyses (JAENCHEN 1996a, VOVK and PROSEK 1997a, 1997b, MALL 1998).

The application and validation of modern TLC are comparable with those of LC (NAGY-TURÁK et al. 1995, RENGER et al. 1995) and the inherent limitations of TLC can only be overcome by the use of hyphenated techniques, *i.e.* any method combined with TLC. SOMSEN et al. (1995) have published a comprehensive survey of the progress made in combining TLC with a variety of spectroscopic techniques. These methods usually lean strongly on the expertise and skills of a chromatographer. The possibility of combining mass spectrometric (MS) techniques with TLC has been introduced by KAISER (1969). The new-generation liquid secondary ion mass spectrometry with TLC (TLC-LSIMS) can determine both mass spectra and mass chromatograms (BUSCH 1992). Because TLC supports are weak Raman scatterers, Raman spectroscopy can also be used as an identification method in TLC (EVERALL et al. 1991). IR spectra are more useful for the identification of unknown substances than UV spectra, because an IR spectrum provides more information about the structure (STAHLMANN et al. 1998). For this reason an HPTLC-FTIR spectroscopic method has been developed (KOVAR et al. 1991, STAHLMANN et al. 1998). TLC with flame ionization detection (TLC-FID) is a promising procedure for the quantitative determination of lipids and other samples that do not absorb UV or VIS (ACKMAN et al. 1990, HWANG and MAERKER 1993). The location and quantification of separated radioisotope-labeled substances requires the use of autoradiography, zonal analysis, or direct scanning with an imaging proportional counter (SHERMA 1994). TLC coupled to high resolution magic-angle-spinning (HR-MAS) solid-state NMR has been introduced in connection with the development of octadecyl silica plates. The solutes are scraped from the layer, slurried with deuterium oxide (D_2O), and analyzed by NMR (WILSON et al. 1997). Atomic absorption spectrometry (AAS) has been used to measure the stability of zinc complexes (ISHII and TAKEUCHI 1989), and TLC-square-wave anodic stripping voltametry (TLC-SWASV) has been introduced for the *in situ* separation of heavy-metal cations (ALDSTADT and DEWALD 1992). Although the use of hyphenated TLC methods is increasing, a considerable amount of work needs to be done on their use with a wide range of solutes, matrices and supports.

Documentation in TLC

Documentation of the TLC/HPTLC plates can be performed by simply storing the original chromatoplate, drawing a copy, making a photocopy or taking a photograph with a 35 mm or polaroid camera for further evaluation. Several commercial, photography-based documentation techniques have been introduced (JORK et al. 1990, VITEK 1991, FRIED and SHERMA 1992, LAYMAN et al. 1995). The advantages, application fields and limitations of colour video technology have recently been compared to conventional colour photography (VÉGH et al. 1998). When maximum reproducibility over a specific UV/VIS range is required the use of conventional photography is preferable. Nevertheless, the capacity of recordable compact discs and packet-writing functions will make these storage devices more attractive in the future.

3.2.2. High-performance capillary electrophoresis (CE)

In general, CE can be considered to be the electron-driven separation of a number of substances in a narrow tube under a high voltage. Even though the separation medium in most applications has been an aqueous solution, the capillary can also be filled with chromatographic packings such as those used in HPLC, the coatings used in GC, or electrophoretic gels. The basic CE methods include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MECC or MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). During the 1990's CE has received an increasing acclaim in the analysis of plant secondary metabolites due to its extremely high efficiency, small sample volume, high speed and good resolution (TOMÁS-BÁRBERÁN 1995).

Capillary zone electrophoresis (CZE) is a powerful technique for the separation of ionic or ionisable compounds. The separation of molecules is based on differences in the charge to mass ratio. The molecules are eluted from the capillary in the order of decreasing positive charge (TOMÁS-BARBERÁN 1995). Electro-osmosis occurs above pH 4, and the electro-osmotic flow (EOF) ensures that positively charged, neutral and negatively charged analytes come out at the cathode end of the capillary. Analytes with smaller mass and greater negative charge will be repelled from the cathode, as will molecules with larger migration times (MT), whilst small positively charged species will emerge from the capillary first. CZE can be achieved with or without complexing agents that can form complexes with electrophoretically neutral molecules like sugars, and these complexes, having a negative charge, can be separated by CE (HOFFSTETTER-KUHN et al. 1991).

Micellar electrokinetic capillary chromatography (MEKC) can separate both neutral and hydrophobic analytes. MEKC is especially useful in enhancing the separation of neutral compounds of similar structure (TERABE et al. 1984), and in separating ionic compounds (KHALEDI et al. 1991, STRASTERS and KHALEDI 1991). In MEKC, knowledge of the hydrophobicity and hydrophilicity of the analytes can assist in selecting the right type of modifier for the electrolyte solution. Ionic surfactants are added to the operating electrolyte above the critical micelle concentration (CMC). The formation of micelles provides a possibility for liquid-liquid differential partitioning of the solute molecules between an aqueous phase and an electro-osmotically pumped, pseudostationary micellar phase. Different types of surfactants, surfactant mixtures and additives to surfactant solutions can be added to the electrolyte to improve the selectivity and specificity of the MEKC separation (BEALE 1998). The most common surfactant is anionic sodium dodecyl sulphate (SDS), *i.e.* sodium lauryl sulphate.

CE has been applied to the analysis of acidic, basic and uncharged plant-derived metabolites. Aromatic phenolic acids were the first secondary metabolites to be analyzed by CE (FUJIWARA and HONDA 1986). Since then there have been numerous applications in the detection of phenolic compounds (BJERGEGAARD et al. 1992, MORIN and DREUX 1993, TOMÁS-BÁRBERAN 1995), but only the applications involving marker techniques have improved the repeatability of analysis and the reliability of identification (SUMMANEN et al. 1995, LIANG 1997, LIANG et al. 1998). Absolute migration times in CZE and MEKC tend to be non-repeatable due to the changes in electro-osmotic velocity (ν_{eo}) and effective electric field strength (E_{eff}). E_{eff} is the net force affecting the velocity of the com-

pound (JUMPPANEN et al. 1993). Marker compounds can be used in CZE to approximate the ν_{eo} and E_{eff}. The information about ν_{eo} and E_{eff} is used to determine the electrophoretic mobilities of the analytes (JUMPPANEN and RIEKKOLA 1995). It might be difficult to determine the true mobilities of the marker compounds due to their partition into micelles in MEKC, but this difficulty can be overcome by introducing migration indices (SIRÉN et al 1994).

3.2.3. Mobile phase optimization in liquid chromatography

Optimization involves the selection of experimental conditions needed to achieve adequate separation and acceptable retention time for each individual sample. Finding the overall optimum conditions requires usually preliminary experiments, and is therefore a compromise between contradictory objectives. An efficient optimization method should be employed during the method development process in order to deal with optimization problems. The optimization can be done manually, statistically or using computer-aided methods.

The "PRISMA" system

The "PRISMA" model has been developed by NYIREDY et al. in order to simplify the optimization process in liquid chromatography (LC) (NYIREDY et al. 1985a,b,c, 1988, DALLENBACH-TÖLKE et al. 1986, NYIREDY 1987). The model is a structured, trial-and-error method and it is based on the Snyder classification of solvents according to their properties as proton donors (X_d), proton acceptors (X_a) and dipole interactions (X_n) (SNYDER 1978). Preliminary experiments are carried out with different solvents from the eight groups in the classification. The "PRISMA" model in TLC consists of three parts: an irregular frustum for selection of basic parameters *e.g.* solvents, a regular middle part for selecting optimal combination of the selected solvents, and a platform. The solvent strength is represented by the height of the prism, points along the edges stand for combinations of two solvents, the points on the sides for combinations of three solvents and the points in the interior of the prism for mixtures of four solvents. Solvent strength and/or incidental tailing of the analytes can be influenced by small amounts of modifiers, symbolized by the lower part of the prism.

The mixtures of mobile phases, *i.e.* the volume fractions of each organic solvent are represented by the selectivity points (P_S) depicted as three-digit numbers. These numbers are obtained by multiplying the volume fractions by 10, and arranging them in order of diminishing solvent strength. The points symbolize quaternary, ternary or binary eluent mixtures. With non-polar samples, the initial solvent composition corresponds to the centre (selectivity point, $P_S = 333$) of the triangular top face of the regular prism. This mixture is diluted with n-hexane to give solvents in the required R_f range. The solvent strength is maintained, and a further three chromatograms are run at solvent compositions corresponding to selectivity points near the apices of the triangle. Based on these initial runs, further chromatograms are performed with different compositions until the best solvent mixture is reached. With polar samples the upper face of the frustum is utilized, and the optimization proceeds in a similar fashion. The last step is the selection of the appropriate development mode. The construction of the model and the role of the solvent

strength and their selectivity points have been extensively described by NYIREDY et al. (1989).

The model has been employed with both isocratic and different gradients in various liquid chromatographic methods for coumarins, cyanobacterial hepatotoxins, flavonoids and phenolic acids (NYIREDY et al. 1986, 1989, ZOGG et al. 1988, VUORELA et al. 1988, 1989, PELANDER et al. 1997, SUMMANEN et al. 1998). Recently, the "PRISMA" optimization system has been used as a basis for a computerized system in both column and planar chromatography (OUTINEN et al. 1996, 1998, PELANDER et al. 1999).

Computer-aided method development in LC

Computer simulations allow the user to investigate a large number of different conditions following a limited number of practical runs, thus providing a rapid return on the cost of the software. Development time is minimized, and is more consistent because a number of variables are optimized simultaneously.

A number of computer-based methods have been used as an aid in HPLC method development (WATSON and CARR 1979, KONG et al. 1980, GLAJCH et al. 1980, GLAJCH and SNYDER 1990). The programs have been designed to help chromatographers optimize separation conditions either by using fewer actual trial runs (*e.g.* DryLab, LC Resources, Lafayette, USA) or by predicting the mobile phase on the basis of the chemical structure of the solutes (*e.g.* EluEx, CompuDrug Chemistry Ltd., Hungary). Expert systems differ from the software in having the ability to process knowledge as well as numerical data by means of heuristic logic. They operate by imitating or simulating the working methods of an analyst *e.g.* Turbo LC Plus Method Development System, Perkin Elmer Solvent Optimization System, Norwalk, Conneticut, USA (GOLGAN and POLLARD 1992).

At the present time, most TLC analyses are still being developed in a nonsystematic manner. Overlapping resolution maps (TECKLENBURG et al. 1984), the sequential simplex procedure (BAYNE and MA 1987), window diagrams (WANG et al. 1989), graphical optimization method (ISSAQ and SEBURN 1989) and an evaluation criterion, *Q*, connected to Snyder´s classification of solvents (CIMPOIU et al. 1998), have been suggested as optimization methods for mobile phase selection in TLC. WANG et al. (1998) have recently developed a computer program for optimizing of mobile phase selectivity. MARKOWSKI (1993, 1996) has introduced a theoretical model as a basis for the optimization in manual multiple development. In spite of this, the optimization in AMD is frequently carried out by trial and error.

The usefulness of different expert systems, alone or combined with software for LC method development, has not been properly tested and published in the field of TLC. A range of desirability functions have been used in separation science to improve the quality of the separations (MORGAN and JACQUES 1978, GLAJCH et al. 1980, OTTO and WEGSCHEIDER 1983, DEMING et al., 1989, DEMING 1991). The aim of this study was to test the usability of the computer aided "PRISMA" model and desirability function concept in determining the optimal selectivity of separations in NP- and RP-TLC ("PRISMA" optimization program, in co-operation with Department of Pharmacy; Division of Pharmacognosy, Department of Mathematics, and Department of Computer Science, University of Helsinki, Finland).

3.3. Biological Assays

Calcium (Ca^{2+}) is an essential cation for many important processes in both intra- and extracellular metabolism (BERRIDGE 1993, CLAPHAM 1995). Calcium homeostasis is regulated by two interdependent cycles (KATZ 1997). Selective or non-selective calcium channels in the plasma membrane control calcium uptake into the cells, while ryanodine (R_y) receptor and inositol-1,4,5-trisphosphate (IP_3) channels are responsible for intracellular calcium fluxes between the cytosol and stores in the sarcoplasmic reticulum. In acute inflammation calcium is a key mediator of activation of the main effector cells, polymorphonuclear leukocytes (PMN) in which calcium influx is controlled by non-selective cation channels (ROSALES and BROWN 1992, KOCH et al. 1994). Selective calcium channels in the plasma membrane are of special importance because they act on the cardiovascular system (KATZ 1997). In this study the biological activity of simple phenolic compounds and *P. emblica* L. leaf extracts was tested by measuring the anti-inflammatory effects in human polymorphonuclear leukocyte (PMN) functions (degranulation, migration, and leukotriene B_4 [LTB_4] release) and platelet activity (aggregation and thromboxane B_2 [TXB_2] production). Calcium transport activity was studied by measuring $^{45}Ca^{2+}$ influx in cultivated rat pituitary CH_4C_1 cells. The whole cell patch-clamp technique was used to selectively study which type of calcium influx mechanism was involved in the measurements. The basic principles involved testing anti-inflammatory activity and importance of calcium in cell metabolism are described in the following.

3.3.1. Anti-inflammatory activity

The first phase of inflammation is caused by an increase in vascular permeability, resulting in the exudation of fluid from the blood into the interstitial space, the second phase by infiltration of leukocytes from the blood into the tissues, and the third phase by granuloma formation. An array of physiological substances, also called autacoids, are involved in the process of inflammation and repair. The discovery of histamine, serotonin, bradykinin, substance P, the group of eicosanoids (prostaglandins, thromboxanes and leucotrienes) and the eicosanoid pathway, the platelet-activating factor (PAF), and cytokines and lymphokines, have resulted numerous *in vitro* studies (VOGEL and VOGEL 1997).

Bradykinin produces pain by stimulating A and C fibers in the peripheral nerves, participates in the inflammatory reaction and lowers blood pressure by means of vasodilatation. The ^3H-bradykinin receptor binding is used to detect compounds that inhibit the binding of ^3H-bradykinin in membrane preparations obtained from guinea-pig ileum. Two types of bradykinin receptors (BK_1 and BK_2 receptors) are known (FERES et al 1992, BASCANDS et al 1993, TROPEA et al 1994). Substance P is released from neurons in the midbrain in response to stress, where it facilitates dopaminergic neurotransmission, and from sensory neurons in the spinal cord in response to noxious stimuli, where it excites dorsal neurons. The selective antagonists to substance P, found in receptor binding studies may elucidate the physiological role of substance P and may be candidates for anti-inflammatory and analgesic drugs (VOGEL and VOGEL 1997).

In acute tissue inflammation, polymorphonuclear leukocytes (PMNs) migrate undirectionally along a chemical gradient of the stimulus (chemotaxis). The acti-

vation of PMN by chemoattractants via a receptor-mediated mechanism, involves the coupling of the agonist/receptor complex with guanosine nucleotide-binding (G) proteins, and the activation of phosphoinositide-spesific phospholipase C, leading to an increase in the intracellular free calcium concentration and activation of protein kinase C (PKC). Stimulation of the signal transduction cascade in phagocytic cells activates the PMNs to migrate, degranulate and produce inflammatory mediators (SNYDERMAN and UHING 1992, EDWARDS 1994). The role of calcium is crucial because it activates or regulates most of the enzymes participating in the signal transduction cascade in PMNs (SMOLEN 1992, SNYDERMAN and UHING 1992, BAGGIOLINI et al. 1993, BOKOCH 1993, MCPHAIL et al. 1993, THELEN et al. 1993). Appropriate assay systems for the determination of different eicosanoids allow studies to be carried out on the influence of drugs on the specific pathways of the arachidonic acid cascade in various cellular systems (VANE and BOTTING 1987). The most commonly performed tests are the formation of leukotriene B_4 (LTB_4) in human white blood cells (PMNs) *in vitro* (VEENSTRA et al. 1988), the formation of lipoxygenase (LO) products from C-14-arachidonic acid in human PMNs *in vitro* (BORGEAT and SAMUELSSON 1979), the formation of eicosanoids such as tromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) from ^{14}C-arachidonic acid in human platelets *in vitro* (POWELL 1985, WEITHMANN et al 1994), and stimulation of the inducible prostaglandin pathway in human PMNs (HERRMANN et al. 1990, WEITHMANN et al. 1994). According to recent findings there are two forms of cyclooxygenase, COX (LEE et al 1992, SMITH et al. 1994, VANE 1994). The inhibition of these cyclooxygenases by the classical cyclooxygenase inhibitors is now generally accepted as an explanation of their adverse side effects. COX-2, which shares about 62% amino acid homology with COX-1, is only expressed after cell activation, especially by mitogenic or inflammatory stimuli (HERRMANN et al 1990, FUNK et al 1991). Thus, specific suppression of the COX-2-pathway may represent a superior target for the evaluation of new, anti-inflammatory drugs.

Free radical (FR) scavenging agents also play a role in inflammation, because liberation of FRs causes tissue damage during the inflammatory process. The scavenging of reactive oxygen species (ROS) has been shown to play an important role in the anti-inflammatory activity of gallic acid and its derivatives (KROES et al. 1992). Flavonoids have profound effects on the functioning of immune and inflammatory cells, as demonstrated by a large number and variety of *in vitro* and *in vivo* observations (MIDDLETON and KANDASWAMI 1992, HARBORNE 1994, FORMICA and REGELSON 1995).

3.3.2. Calcium fluxes in cell metabolism

Calcium channels are present in different tissues, such as in the endocrine and nervous systems, and cardiac and smooth muscles. In addition, calcium serves a second messenger function for the action of many hormones. One major role of calcium regulation is activation of the intracellular calcium cycle, which in turn releases calcium that initiates excitation-contraction coupling. Regulation of the calcium concentration is under tight endocrine control, affecting its entry at the intestine and its exit at the kidney, and regulating the large skeletal reservoir for withdrawal in times of need.

The cytosolic Ca^{2+} concentration can rise to a higher level in many illnesses. A high Ca^{2+} concentration can provoke the development of insufficiency, and finally an infarct (LI et al. 1995, KATOPODIS et al. 1997). In chronic kidney insufficiency, the rise in intracellular Ca^{2+} is connected to disturbances in fat metabolism (KLIN et al. 1995, NI et al. 1995). Furthermore, findings of disturbances in the intra-/extracellular Ca^{2+} ratio support the importance of calcium balance in breast and prostatic cancer (GONG et al. 1995, NIE et al. 1997), epileptic attacks (VAN LUIJTELAAR et al 1995), and mental disorders (TAN et al. 1990, JOFFE et al. 1996, ERESHEFSKY et al. 1996). A high dietary intake of calcium lowers the intracellular free calcium $[Ca^{2+}]_i$ (SAITO et al. 1995). Calcium supplement has been found to play a crucial role in the prevention of osteoporosis (MURRAY 1996), as well as in other illnesses e.g. elevated blood pressure (SAITO et al. 1995). Calcium channel antagonists (CCAs) have conventionally been used in the treatment of cardiovascular disorders. Epidemiological surveys and clinical studies also support their therapeutic usability in other disorders, such as kidney failure (TIKKANEN and JOHNSTON 1997), epilepsy (VAN LUIJTELAAR et al 1995) and breast cancer (CHARLIER et al 1996).

Three types of Ca^{2+} channels regulate the calcium level at the plasmalemma; voltage-dependent, receptor-operated (ROCCs) and stretch operated channels. The L-, N-, P-, and T-type channels at least are involved in controlling the voltage operated calcium channels (VOCCs). The L-type (slow) and T-type (transient) channels are located in the heart and smooth muscle cells, the N-type channels are found only in neuronal tissues, and the P-types are located in the Purkinje cells. CCAs inhibit Ca^{2+} influx from the extracellular space into the cytosol by binding at various sites on the L-type channels (KATZ 1997). The other channel types are apparently not sensitive to CCAs.

Calcium channel blocking activity can be determined by measuring the inward calcium current through the calcium channels. However, test models can only be a first step in determining a possible calcium channel blocking effect. Test models comprise animal experiments, experiments with isolated muscle preparations, and experiments with cell lines or cultured cells (VUORELA et al. 1997). The patch clamp technique provides the experimental means for analyzing the exact mechanisms of calcium channel modulation. Candidate messengers can be tested directly on excised patches or in whole-cell recording (CAHALAN and NEHER 1992).

One suitable model for testing a prospective calcium antagonist is smooth-muscle preparations of the rabbit or rat aorta. Contractions of the aorta are evoked by K+ depolarization, which selectively opens VOCCs to allow extracellular Ca^{2+} into the cytosol; calcium channel blockers inhibit these contractions (HOF and VUORELA 1983, SPEDDING and CAVERO 1984). The cells of the pituitary gland have been found to possess VOCCs (OZAWA and KIMURA 1982) that can be stimulated by thyrotropin-releasing hormone (TRH) and by depolarization with high external potassium. This means that rat pituitary CH_4C_1 cells can be used as a model for studying compounds that interact with calcium channels. The following criteria can be used for choosing plant extracts or substances for the biological testing of calcium-antagonistic activity: screening of plants used to treat cardiovascular disease in traditional medicine, idiosynchrony-guided screening, *e.g.* non-specific spasmolytic activity in which the mode of action is not clearly shown, screening of natural compounds structurally related to known CCAs, and chemotaxonomy-guided chemical and pharmacological screening (VUORELA et al. 1997). Since CCAs are in wide clinical use as therapeutic agents, some screening

programs for plant extracts have been established (YAMAHARA et al. 1985, VUORELA 1988, ICHIKAWA et al. 1989, RAUWALD et al. 1994). As a result of these studies, naturally occurring substances with possible calcium-antagonistic activity have been found in a range of secondary product groups such as alkaloids (YANO et al. 1991, MARTIN et al. 1993), coumarins (VUORELA et al. 1988, HÄRMÄLÄ et al. 1992, RAUWALD et al. 1994), lignans (ICHIKAWA et al. 1986), phenylpropanes (HWANG et al. 1987, NEUHAUS-CARLISLE et al. 1993, 1997, SENSCH et al. 1993) and flavonoids (MORALES and LOZOYA 1994, SUMMANEN et al. 1999). The compounds that show selective activity in VOCCs in the testing systems can be regarded as models in the search for new CCAs.

4. Experimental

A detailed presentation of the materials and methods can be found in the original publications.

4.1. Materials

Phenolic compounds

Table 3. *Sources of the simple aromatic compounds, coumarins and flavonoids used in this study.*

Compound	**Source**
Phenylmetanes	
Benzoic acid	Merck, Germany
Butylated hydroxyanisole (BHA)	BDH Laboratory Chemicals Division, UK
Butylated hydroxytoluene (BHT)	Sigma, MO, USA
Gallic acid monohydrate	Sigma, MO, USA
Methyl gallate	Sigma, MO, USA
n-Dodecyl gallate	Fluka, Switzerland
Gallic acid trimethyl ether	Sigma, MO,,USA
Gallic acid amide	Lancaster, UK
n-Octyl gallate	Sigma, MO, USA
n-Propyl gallate	Sigma, MO, USA
Syringic acid	Roth, Germany
Phenylpropenes	
Caffeic acid	Roth, Germany
Ferulic acid	Sigma, MO, USA
Flavonoids	
- Flavones	
Apigenin	Roth, Germany
Luteolin	Roth, Germany
Acacetin	Roth, Germany
Flavone	Roth, Germany
Vitexin	Roth, Germany
Vitexin-2´´-O-rhamnoside	Roth, Germany
Luteolin-7-glucoside	Roth, Germany
Luteolin-3´,7-glucoside	Roth, Germany
- Flavonols	
Quercetin	Roth, Germany
Rhamnetin	Roth, Germany

Compound	Source
Isorhamnetin	Roth, Germany
Morin	Roth, Germany
Quercitrin	Roth, Germany
Rutin	Roth, Germany
- Flavanones	
Naringenin	Roth, Germany
Naringin	Roth, Germany
- Isoflavones	
Daidzein	Roth, Germany
Genistein	Roth, Germany
Daidzin	Roth, Germany
Genistin	Roth, Germany
Coumarins	
Herniarin	Roth, Germany
Scopoletin	Roth, Germany
Umbelliferone	Roth, Germany
Other phenolic compounds	
Ellagic acid	Sigma, MO, USA

Plant material

Leaves of *P. emblica* L. (Euphorbiaceae) were collected from a wild tree close to the beach of Teluk Bahang, Penang Island, Malaysia. The plant was identified by Prof. Mashhor Mansor, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia, and a voucher specimen (No. 6023) was deposited in the herbarium of the School of Biological Sciences, Universiti Sains, Malaysia, Penang, Malaysia. The leaves with their petioles were oven dried at 40°C for 48 hours and kept in paper sacks in dry and dark conditions at room temperature until used.

4.2. Methods

Extraction of the plant material

Ten solvents of different selectivity were chosen for the extraction procedure according to their ability to extract compounds over the whole R_f range with reference to their biological activity (SNYDER 1978, HÄRMÄLÄ et al. 1992). Details of the semiquantitative TLC separation are given in **V**.

Preparative chromatography

The preparative chromatography was carried out using RP medium pressure liquid chromatography (MPLC), using LiChroprep RP-18 phase material (YRJÖNEN et al., unpublished results).

Analytical CE and TLC methods

Optimization method. The "PRISMA" model described by NYIREDY et al. (1988, ZOGG 1989) was the optimization method applied in all of the TLC separations (**I–V**).

Biological tests for anti-inflammatory activity

The *P. emblica* L. leaf extracts were tested for their inhibitory activity against human polymorphonuclear leukocyte (PMN) functions such as degranulation, migration, leukotriene B_4 release (LTB_4) and platelet activity (aggregation and thromboxane (TXB_2) production). Details of these assay methods are given in **V** and by KANKAANRANTA 1995.

Biological test for calcium uptake studies

The calcium antagonistic activity was measured as inhibition of depolarization-induced uptake of $^{45}Ca^{2+}$ in rat pituitary CH_4C_1 cells as presented. The therapeutically used calcium channel antagonist (CCA) verapamil hydrochloride served as a standard. The method has been described in detail in (**VI**) and by TÖRNQUIST and TASHJIAN (1989). Details of the whole-cell patch-clamp technique are given by CAHALAN and NEHER (1992).

Statistical methods

The calculations and statistical evaluations were performed using Statview SE+Graphics™ for Macintosh, and Systat 6.0 for Windows 95 (**I-VI**). Cluster analysis was carried out using ward linkage with Pearson correlation. In factor analysis the common factor model was selected and the matrix correlation types with Varimax rotation were used (**II**). For the desirability functions the data processing was done by MATLAB (Mathworks Inc, Sherbon, MA, USA) using the Data-Analysis Toolbox (ProfMath, Helsinki, Finland) operating on MATLAB. A windowed graphical user interface was built for data handling, desirability function selection and graphical inspection of the response surfaces (**IV**).

5. Results and Discussion

5.1. Development of Analytical Chromatographic Methods for Plant Phenolics

5.1.1. Analysis of phenolic antioxidants by CE (I)

Various gas chromatographic (PAGE and KENNEDY 1976, AUSTIN and WYATT 1980, WYATT 1981) and high performance liquid chromatographic (ANDERSSON and VAN NIEKERK 1987, ANNAPURANI 1989, ANDRIKOPOULOS et al. 1991) methods have been developed for the determination of phenolic antioxidants. However, each method has its limitations and therefore the search for better analytical alternatives is reasoned. A useful, rapid, routine capillary electrophoretic method (CZE or MEKC) was needed for the analysis of phenolic compounds such as gallic acid, six derivatives of gallic acid, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). The repeatability of the chosen CE method was tested using two carboxylic acids as marker compounds for calculating the migration indices of the analytes. The optimized method was applied to test the purity of compounds extracted from plants, as a reference method for thin-layer chromatography (TLC) by exploiting the high resolution of CE for reliable identifications, and for conformational determinations.

Separation by CZE. CZE was initially employed in the development of this analytical method because of its simplicity, and because the separation mode depends on the molecular properties of the analytes. Different electrolyte solutions were tested to find the optimal separation conditions. The buffer electrolytes and concentrations were 30 mM and 50 mM phosphate (both at pH 7.00), MOPS (3-[N-morpholino] propane sulphonic acid, pH 7.00) and CAPS (3-[cyclohexylamino]-l-propane sulphonic acid, pH 10.60), respectively. However, sufficient resolution could not be obtained.

Separation by MEKC. When the resolution obtained by CZE proved unsatisfactory, MEKC was investigated as an alternative method. MEKC offers better flexibility and selectivity for compounds with similar values of electrophoretic mobility.

The best separation of all the analytes was obtained with sodium dodecyl sulfate (SDS) micelles in a 30 mM phosphate buffer. Different concentrations of SDS micelles in 30 mM phosphate electrolyte solution (pH 7.00) were studied to optimize the conditions for repeatable separations of the phenolic antioxidants. Concentrations of 10, 15, 20, 25, and 30 mM SDS were tested. The 30mM concentration was finally chosen because good separation was achieved for all analytes within 15 min.

Instrumental parameters. The influence of instrumental parameters (capillary length and applied voltage) on the separation of 9 phenolic antioxidants was studied. Uncoated fused-silica capillaries with detection lengths of 49-53 cm were tested. Voltages of 16, 18, 20, 22, and 24 kV were used with each capillary length.

The best resolution was obtained with a 51-cm capillary (total length 59.5 cm) and a voltage of 22 kV.

Repeatability and reliability. The repeatability of the separation was studied by applying the two-marker technique developed for CZE to MEKC. Potential problems due to partitioning of the marker compounds into micelles were avoided through the use of migration indices.

The reliability of the identification can be expressed by using identification coefficients Q_{id}, which can be calculated for successive peak pairs. The responses were both the absolute migration times and the migration indices. If the Q_{id} value exceeds 2, the identification between two compounds is considered reliable (see Table 3, **I**).

Purity testing. The suitability of the optimized MEKC method for investigating the diversity of three *Phyllanthus emblica* L. medium-pressure LC fractions was examined. The preliminary TLC tests showed that the main compound in fraction 2 was probably a compound of medium polarity. MEKC proved to be suitable for the rapid testing of plant extracts for compounds of similar chemical character, such as gallic acid derivatives. An optimized phosphate-SDS electrolyte solution in MEKC provided sufficient selectivity for the satisfactory resolution of gallic acid and the 6 derivatives of gallic acid, BHA, and BHT. The SDS concentration was especially important because the main structures of the analytes were similar. The separation could be carried out within 15 min. The electrophoretic mobilities increased as the size of the alkyl group on C2 increased, as follows:

$$OCH_2 > O(CH_2)_2CH_3 > O(CH_2)_7CH_3 > O(CH_2)_{11}CH_3.$$

In addition, the methyl groups in the C3, C4, and CS positions at the benzene structure increased the partition of the analytes into the micelles.

5.1.2. AMD (Automated Multiple Development) optimization (III)

Phenolic compounds are usually tightly adsorbed to the silica gel, leading to a need for numerous experiments to determine the optimal separation conditions. The AMD technique and its suitability for the separation of various complex mixtures enables real reproducible gradient elution and increased separation power on TLC plates. Both gradient development and the band reconcentration effect act in the direction of improving the spot capacity. A succesful separation depends mainly on choice of the solvent components, the steepness of the gradient, and optimization of the shape of the gradient. Systematic experiments were therefore performed to evaluate gradient elution in Automated Multiple Development (AMD) for finding a suitable gradient system for the separation of a phenolic reference mixture (±catechin, ellagic acid, gallic acid methylester, gallic acid monohydrate, kämpherol, and rutin).

The development time, number of development steps, drying time between each run, amount of high polarity and medium polarity solvents, amount of acid as a modifier during the development procedure, and pre-conditioning of the vapour space and the preconditioning time of the silica gel TLC plates, were the instrument parameters tested to evaluate the optimal conditions for AMD separation.

On the basis of the preliminary isocratic TLC experiments, the best separation on the HPTLC plate with an ordinary ascending run was achieved using a solvent

mixture of toluene, ethanol, and formic acid with a selectivity point Ps 3 6 1. The solvent strength (S_T) was adjusted with n-hexane to 2.4, resulting in a mobile phase of 60 % toluene, 18 % ethanol, 4 % formic acid and 18 % n-hexane. Neither rutin nor ellagic acid could be separated totally from the other compounds with this solvent mixture.

Acetone and dichlormethane were chosen for the solvent system for further optimization of the other parameters in this study. Because no differences were found between prewashed and untreated TLC strips in the AMD development, the TLC strips were used as such. 5 cm x 10 cm HPTLC aluminium plate strips were used instead of 10 cm × 20 cm glass plates. The strips were attached to an acid-resistant steel sheet specially developed for this study. The separation obtained on the TLC strips was repeated with 10 cm × 20 cm glass plates, but no differences were found between these two types of plate.

Development time. Increasing the development time from 110 min to 220 min also markedly improved the separation of the reference compounds. However, slight band broadening was seen when the development time was gradually increased from 110 min to 220 min.

Drying time. When the development time was kept at 220 min and the number of development steps at 25, neither increasing nor shortening the drying time caused any further broadening or tailing of the bands, except for rutin, the peak width of which was doubled in the case of the shortest drying time. The shortest drying time improved the separation of the compounds with lower Rf values, ellagic acid and rutin.

Number of development steps. Increasing the number of development steps from 10 to 25 resulted in an improvement in the separation of the reference compounds especially after 15 development steps, but the band broadening of gallic acid methylester increased. Band broadening for ellagic acid and rutin decreased markedly, making a clear separation of all six compounds possible at 20 development steps.

Proportion of high polarity and medium polarity solvent. Reducing the proportion of the polar solvent, acetone, from 100 % to 25 % in the first development step with 10 development steps gave a slightly better separation, although ellagic acid and rutin could not be eluted from the starting zone. A slight increase in band broadening was also observed.

Proportion of modifier. Separation of the compounds was slightly improved when formic acid was kept in the solvent system from step 1 to step 20. Omitting formic acid from step 2 and from the remaining steps caused more band broadening compared to the situation in which acid was included.

The best separation of ±catechin (C), ellagic acid (E), gallic acid methylester (GM), gallic acid monohydrate (G), kämpherol (K) and rutin (R) was achieved with 20 development steps, N_2 bubbled for 15 s through 10 % formic acid as the conditioning atmosphere in each step, and a drying time of 2 minutes after step one followed by 3 minutes for the remaining 19 steps (see **Fig. 12**, **II**). The suitability of the method for screening purposes was tested with a plant extract of *P. emblica* L. (Euphorbiaceae) leaves (see **Fig. 13**, **II**). According to the reference UV spectra the methanol extract contained a gallic acid derivative (GD). Drying times shorter than 1.0 min at step one and 1.5 min at the remaining development steps improved the separation of the analytes, but caused considerable band broadening especially for rutin (R). An acidic modifier had to be added to the eluent system in order to achieve satisfactory separation.

5.1.3. Evaluation of video documentation and densitometer parameters in the detection of plant phenolics by TLC (III)

Documentation of planar chromatography must fulfil several criteria to be suitable for analytical purposes. In recent years commercially available detection methods using color-video technology have increased the versatility of TLC. Video documentation can collect useful information from the complete plate more quickly than more commonly used densitometer and instant photography techniques (VOVK and PROSEK 1997a, VÉGH et al. 1998). A comparison between densitometer and video documentation systems was carried out by testing the influence of a range of parameters in these systems. Phenolic test mixture no. 1 was a mixture of phenolic acids and flavonoids consisting of ± catechin, ellagic acid, gallic acid monohydrate, methyl gallate, kämpherol, and rutin. Test mixture no. 2 consisted of three coumarins, herniarin, scopoletin, and umbelliferone. The settings tested, and those kept constant, are listed in **Table 3**.

Table 3.

Tested densitometer and video documentation system parameters

Shimadzu CS-9001 PC Densitometer	Camag Video Documentation System with Reprostar 3 – CCD HV-C20 camera
– Beam size (mm; 0.4 × 0.4, 0.2 × 1.0 or 1.0 x 5.0) – Data accumulation (1 or 4) – Reflectance mode – Lamp mode UV 254 nm or 366 nm	– DTL: off, low, normal or high (" resolution settings") – Frame accumulation (on or off), – Camera integration (frames; 1, 2, 3 or 5, 1 frame = 40 ms) – Aperture (f; 2, 2.8, 4, 5.6, 8 or 11)
Parameter modes that were kept constant	
– Linearizer : Off – Y axis resolution: 0.10 – Scan type: Linear – Scan mode: Normal – Zero mode: At start	– Camera mode: Manual – White balance: Auto – AGC: Off, Limit: + 18 dB – Gain: Off, Level: + 9dB – White balance: Auto – Shutter: Off, Speed: 1/100 sec – Gamma: Off – Contrast: off – Shade mode: Lumin

For NP separation a mixture of 60 % toluene, 18 % ethanol, 18 % n-hexane and 4 % formic acid was used for test mixture No. 1, and a mixture of 23 % tetrahydro-

furan, 2 % 2-propanol and 75 % n-hexane for test mixture No. 2. For RP separation the solvent system for test mixture no. 1 was 50 % methanol and 50 % water with 1 % of o-phosphoric acid as a modifier. The best separation by RP of test mixture no. 2 was achieved using 37 % tetrahydrofuran, 5 % 2-propanol, 5% acetonitrile and 53 % water. The ethyl acetate phase of the methanolic leaf extract of *P. emblica* L. served as an example of plant material containing phenolic compounds.

Influence of parameters. In both the NP and RP separation systems for phenolic acids and flavonoids (test mixture no. 1) the DTL setting had an effect on the quantative result, i.e. on the mean and the calibration curve (SDV%). Frames (integration period), aperture and image accumulation interacted with reproducibility (coefficient of variation, CV%). Sufficient light and the exposure period were thus important variables needed to obtain good analysis results. The parameter interactions for test mixture no. 2 using NP separation show that aperture was connected to reproducibility (CV%), to image accumulation and to DTL. These were together connected with the mean of the measurements and the integration period. In the RP measurements, image accumulation was linked with the calibration curve (SDV%), and were dependant on the two parameter groups: the integration period and mean of the measurements, and the aperture, reproducibility (CV%) and DTL.The results of factor analysis supported the results obtained with cluster analysis (see **Fig. 1**, **III**).

Repeatability. The tracks of the standards were detected six consecutive times with the densitometer and with the video scanning function.

Umbelliferone. When NP was used as the stationary phase and the chromatograms detected at 366 nm, the coefficient of variation (CV %) was 1.9 % for the densitometer and 1.5 % for the video scanning system. The CV % was 1.9 % for the densitometer and 0.5 % for the video scanning system when detection was carried out at 366 nm using RP as the stationary phase.

Methyl gallate. The CV % at 254 nm was 0.4 % for the densitometer and 3.8 % for the video scanning system when using NP plates as the stationary phase. When RP was used the results were 0.9 % and 1.5 %, respectively. When frame accumulation was set in the off position, the coefficient of CV% values were markedly lower than with the on position.

The smaller the chosen aperture (f) value, the higher was the regression coefficient values (R^2) for test mixture no. 2. Smaller aperture values also had a decreasing effect on the CV% values. The results obtained with the densitometer proved to be better when analysing methyl gallate on NP, but the situation was the opposite on RP plates. However, both methods gave similar, satisfactory results.

Comparison of repeated measurements of quantitative amounts of reference compounds (n = 6). According to the regression coefficients (R^2) of the calibration curves, these two methods can be considered equal (see **Table 4**). The regression coefficients for umbelliferone calculated from the densitometric measurements were slightly better than those obtained by the video documentation system. According to these results, a chromatogram made from the taken image can give as satisfactory information as a densitogram, but the densitometer is slightly more sensitive than the video documentation system. Nevertheless, the densitometric technique may be more suitable when UV wavelengths other than 254 nm and 366 nm are needed. Quantitative determination was carried out using an external standard for calibration. The Rf value of gallic acid derivative (GD) was close to that of gallic acid monohydrate in sample mixture no. 1. The UV spectrum of gallic acid derivative (GD) was very close to that of gallic acid monohydrate (GA)

and methyl gallate (MG), and therefore it may have a similar structure to that of GA or MG. For this reason the quantitative determination of this unidentified compound was based on the calibration data for methyl gallate. The calibration curve was calculated from eight measurements of the reference substance (methyl gallate). The standard deviation (SD) and relative standard deviation (RSD %), which express the repeatability, are presented in **Table 5**.

***Table 4**. Comparison of the regression coefficients (R^2) of the calibration curves for methyl gallate at 254 nm and umbelliferone at 366 nm in normal (NP) and reversed phase (RP) separations.*

Compound	Shimadzu CS-9001 PC Densitometer, R^2		Camag Video Documentation System with Reprostar 3, R^2	
	NP	**RP**	**NP**	**RP**
Methyl gallate	0.984	0.990	0.990	0.986
Umbelliferone	0.974	0.988	0.945	0.937

***Table 5.** Repeatability. Calibration with the equation for methyl gallate.*

Measurement no.	Amount gallic acid derivative (GD, µg/spot) RP-18 F254s plate	
	Densitometer	**CCD camera**
1	0.553	0.583
2	0.482	0.420
3	0.505	0.438
4	0.556	0.398
5	0.493	0.463
6	0.479	0.452
Mean	0.511	0.459
SD	0.035	0.065
RSD (%)	6.78	14.16

Both data acumulation in densitometric evaluation and frame accumulation with the Camag video documentation system entail a long integration feature and effectively increase the possibility of capturing weaker spots on the TLC layers. Both video and densitomettric methods are suitable for analysts lacking skills to make documents of TLC plates. Independence of the mode of separation and the structure of the TLC layer used, the speed of evaluation, and the archiving the captured images for reporting purposes can be seen as advantages of the video documentation. The limitation of both reflectance densitometry and the video camera is the detection of compounds distributed vertically inside the depth of the layer. Further developing of contemporary video cameras for analytical purposes might improve the sensitivity.

Results of the preparative chromatography (YRJÖNEN et al., unpublished results).

Phyllanthus emblica L. leaves were collected, prepared and stored as described in **4.1**. (Materials). Exhaustively milled leaves and petioles were extracted with methanol and the combined extracts were reduced in volume. After the addition of

water and evaporation the residual of methanol the aqueous phase was defatted with petroleum benzene and then extracted with ethyl acetate followed by butanol. The ethyl acetate fraction was further separated by medium pressure chromatography (MPLC, Büchi no. 17982 and 17988, Switzerland) using reversed phase (RP-18) and stepwise gradient elution from water to methanol with 1 % formic acid as a modifier. The repetitive fractionation of the ethyl acetate extract yielded seven components. Structures of rutin, quercetin and gallic acid monohydrate were confirmed by structure elucidation with nuclear magnetic resonance (NMR) spectrometry and four yet unknown compounds.

5.1.4. Optimization of separation in TLC using desirability functions and mixture designs according to "PRISMA" (IV)

The computer software for the optimization of HPLC separations by the desirability function technique and the "PRISMA" mixture design model was applied for optimizating the TLC separation of cyanobacterial hepatotoxins on normal phase (NP) TLC plates, and for phenolic compounds on reversed phase (RP) TLC layers. In order to determine the best separation over the given solvent mixtures, statistical modelling was created for predictive models of the retardation (k_p) and band broadening (peak width, w_h) for each analyte component. Three organic solvents were used as the explanatory variables for the test analyte mixtures. The other experimental conditions were kept constant. As the sum of the mixture components always equals one, the models may be written in terms of only two of the solvents. Possible candidate types for the models are therefore the linear model

$$y = b_0 + b_1x_1 + b_2x_2 \quad (1)$$

or the full quadratic model

$$y = b_0 + b_1x_1 + b_2x_2 + b_{1,1}\,x_1^2 + b_{1,2}\,x_1\,x_2 + b_{2,2}\,x_2^2 \quad (2)$$

An overall optimal selectivity point is a compromise between contradicting objectives: an improvement in the separation for certain components often means a deterioration with respect to some other pairs. There might be no objective "best" point, but the investigator must weigh the various factors in a more or less subjective manner. The desirability functions can be used to enhance the effectivity of the optimization system. Various functions may be used for the desirability profiles, which convert the response (Y) into the desirability value (D):

$$0 < D(Y) < 1 \quad (3)$$

Here 0 indicates a completely unsatisfactory result, whereas 1 indicates that the required level of response has been reached. The desirability functions can be tailored according to the requirements set for the parameter of interest. In this case, the logistic desirability function for the resolution (R_S) was

$$D(R_S) = 1 / 1 + e^{(RSRS0)/d} \quad (4)$$

where the "mean resolution value" R_S0 and "deviation", d, were selected individually according to the chromatographic behaviour of the toxins and phenolic compounds.

Selection of solvent strength and selectivity points. According to the preliminary experiments explained in detail by PELANDER et al (1997), ethyl acetate (EtOAc, S_T=4.4), propan-1-ol (*n*-PrOH, S_T =4.0), and water (H_2O, S_T =10.2) and 5 % acetic acid (S_T =6.0) as a modifier, were selected for the optimization of NP-TLC separation of cyanobacterial hepatotoxins. The experiments were performed in the irregular part of the PRISMA model and the following selectivity points were chosen for the experiments: P_S = 163, 136, 343, 334, 154, 145, 2.5 4.5 3, 2.5 4 3.5, 2.5 3 4.5, 253, 244, 235, 1.5 5.5 3, 1 4.5 4.5, 1.5 4 4.5, and 1.5 3.5 5. Based on the preliminary experiments, propan-2-ol (2-PrOH, S_T = 4.2), acetonitrile (MeCN, S_T = 3.2) and methanol (MeOH, S_T = 2.6) were selected as the organic solvents for the separation of phenolic compounds in RP-TLC. To improve the peak shape, 1 % formic acid was added to the eluent mixture as a modifier. The solvent strength (S_T) was first adjusted to the level S_T = 1.3 with water (S_T =0). At constant S_T the elution combinations described by the selectivity points (P_S) were systematically changed. The following selectivity points were chosen for the experiments: P_S = 811, 181, 118, 631, 361, 163, 136, 316, 613, 433, 343 and 334, 5 2.5 2.5, 2.5 52 .5, and 2.5 2.5 5.

The number of trial runs made on cyanobacterial hepatotoxins and phenolic compounds were 16 and 15, which is sufficient for drawing a statistically reliable conclusion with respect to the number of parameters in the quadratic model (**Eqn. (2)**).

Regression models for retardation and band broadening. After adjusting the solvent strength and testing the experimental selectivity points, the retention data were entered into the computer program for the optimization process. Retardation was expressed as the planar retention factor, $k_p = 1/R_f-1$, where R_f is the measured retardation factor for each compound. Band broadening was expressed as the peak width at half height (w_h). The goodness of fit of the regression model was measured with the coefficient of determination values (R^2). Satisfactory R^2 values were not obtained with linear models, whereas the full quadratic regression models proved to be the best for describing the dependence between the k_p values and P_S (**Eqn.2**). The NP and RP materials produced similar retardation behaviour with regard to the obtained models. In order to measure R_S the the band width (w_h) must be measured for the optimization program. A simple regression giving the w_h as a function of a time was fitted to all of the band width data. The behaviour of w_h on NP and RP materials is shown in **Fig. 6a** and **Fig. 6b, IV**. The cyanobacterial hepatotoxins showed no measurable dependence on k_p (R^2=0.249) whereas the phenolic compounds on RP showed a good dependency on k_p (R^2=0.930). The experiments showed that a regression model for phenolic compounds could be constructed for the determination of R_S but for the toxins a constant (mean w_h value) must be inserted to R_S equation.

Optimization of the selectivity by desirability functions. In order to find an optimum eluent mixture for the separation of cyanobacterial hepatotoxins and phenolic compounds, the resolutions should be predicted at other selectivity points and the overall desirability (D_0) then defined over all the individual target profiles (**Eqn. 5**).

$$D_0 = (d_1\, d_2, \ldots d_m)^{(l/m)} \qquad (5)$$

The program calculated the overall desirability (D_0) on the basis of the data from 16 experimental points for cyanobacterial hepatotoxins. Overall desirability (D_0) surfaces can be depicted as a contour plot inside the horizontal plane of the

"PRISMA". The program suggested three optimum areas for the cyanobacterial hepatotoxins, the best area (D_0=0.9) is located in the right side of the middle point of the prism (see **Fig. 7a, IV**). The best result was achieved regardless of the band width, which was set as constant. In this case the separation became emphasized over the peak width, and some band broadening was observed in the optimized separation.

The overall desirability (D_0) for the runs of phenolic compounds was based on 15 experimental points. The best area (D_0=0.1) was located close to the right side of the middle point of the prism, and the corresponding selectivity point, 2.5 5 2.5, gave a good separation of the phenolic compounds (see **Fig. 7b, IV**).

5.2. Biological Activity

5.2.1. Anti-inflammatory activity of leaf extracts of Phyllanthus emblica *L. (V)*

Leaves of *P. emblica* L. were extracted with ten different solvents from eight different selectivity groups (*n*-hexane, diethyl ether, methanol, tetrahydrofuran, acetic acid, dichloromethane, 1,4-dioxane, toluene, chloroform, and water) according to their ability to extract compounds over the whole R_f range with reference to their biological activity. The effects of the extracts against human polymorphonuclear leukocyte (PMN) and platelet functions were studied.

Effects on PMN functioning. The methanol, tetrahydrofuran, and 1,4-dioxane extracts (56 pg/ml) were the most potent inhibitors (about 90 % inhibition) of the LTB_4-induced migration of human PMNs (see **Fig. 2 A**, **V**). The inhibition of human PMN migration by the methanol, tetrahydrofuran, and 1,4-dioxane extracts was dose-dependent (IC_{50} 13 -15 pg/ml). There was a significant correlation between the inhibition of PMN migration and the relative amount of polar compounds in the *P. emblica* extracts ($r = -0.74$; $P < 0.05$; see **Fig. 2A** inset, **V**), *i.e.* the higher the proportion of polar compounds in any particular extract the better it inhibited PMN migration.

Effects on platelet functioning. The diethyl ether extract (50 µg/ml) inhibited calcium ionophore A23187-induced leukotriene B release from human PMNs by 40 %, thromboxane B2 production in platelets during blood clotting by 40 % (see **Fig. 3 A**, **V**), and adrenaline-induced platelet aggregation by 36% (see **Fig. 3 B**, **V**). Ellagic acid, gallic acid and rutin, all compounds isolated earlier from *P. emblica*, could not explain these inhibitory activities on PMNs or platelets by the *P. emblica* extracts.

Effects of ellagic acid, gallic acid and rutin on human PMN and platelet functions. Ellagic acid, gallic acid and rutin, earlier isolated from *P. emblica*, have been reported to possess some properties indicative of possible anti-inflammatory activity and are commercially available (KROES et al. 1992, WAGNER and DORSCH 1992). None of these compounds (50 pg/ml) affected either FMLP or A23187-induced degranulation of human PMNs (see **V**, **Table 2**). Instead, gallic acid methyl ester (50 pg/ml) was a potent inhibitor of LTB4 release from human

PMNs. It also reduced platelet aggregation and TXB_2 production. Ellagic acid was found to increase TXB_2 production significantly (see **V**, **Table 2**). Neither ellagic acid nor rutin inhibited any of the PMN or platelet functions studied.

The chemical compounds so far isolated from the leaves of *P. emblica* are polyphenolic constituents *i.e.* gallic acid, ellagic acid, chebulic acid, chebulagic acid, chebulinic acid (THERESA et al. 1965, 1967). The inhibitory effects of gallic acid on both 5-lipoxygenase (leukotriene production) and cyclooxygenase (thromboxane production) have been reported earlier and the anti-oxidative and anti-carcinogenic properties of ellagic acid are known (ZEE-CHENG and CHENG 1986, WAGNER and DORSCH 1992). Rutin has been reported to stimulate cyclooxygenase activity (ALCARAZ and FERRANDIZ 1987). According to the TLC experiments, the methanol extract of *P. emblica* does not contain significant amounts of ellagic acid or gallic acid methyl ester, whereas some rutin or gallic acid monohydrate may be present. However, rutin and gallic acid monohydrate did not have any effect on PMN degranulation. The study shows that the leaves of *P. emblica* have antineutrophil and antiplatelet properties *in vitro*, which confirm the antiinflammatory and antipyretic properties of this plant as suggested by its use in traditional medicine by rural populations in Asia. Furthermore, our results suggest that the plant leaves contain as yet unidentified compound(s) with potent antineutrophil activity, and a chemically different molecule(s) either with a dual cyclooxygenase and 5-lipoxygenase or phospholipase A_2 inhibitory activity.

5.2.2. Effects of simple aromatic compounds and flavonoids on calcium fluxes in clonal rat pituitary GH_4C_1 cells (VI)

Calcium channel antagonists (CCAs) act mainly on the slowly deactivating, low activation threshold (L-type) voltage operated calcium channels (VOCCs), inhibiting Ca^{2+} entry to result in the relaxation of vascular smooth muscle (KATZ 1997).

Inhibitory effect on $^{45}Ca^{2+}$ uptake. The calcium antagonistic effect of 9 phenylpropanes and - metanes, and 20 flavonoids was investigated by measuring the depolarization-induced $^{45}Ca^{2+}$ uptake in GH_4C_1 rat pituitary cells. The clinically used CCA verapamil hydrochloride served as a reference compound.

Measurements made with the phenolic compounds indicated that flavones luteolin and flavone had a clear calcium antagonistic activity similar to the therapeutically used CCA verapamil hydrochloride. At the highest studied concentration (20 µg/ml) flavone inhibited $^{45}Ca^{2+}$ entry by 63.5 %, luteolin by 51.4 %, the flavanone naringenin by 56.3 % and the isoflavone genistein 54.6 % (see **Fig. 1** and **Table 1**, **VI**). The phenylmethane derivatives dodecyl gallate and octyl gallate were the most effective CCAs of the tested phenylmethanes showing a 40.4 % and 92.2 % inhibition at 20 µg/ml, respectively. Octyl gallate was the most potent compound tested, with an IC_{50} of 15.0 µg/ml. The IC_{50} for verapamil hydrochloride was 3.0 µg/ml. The phenylmethanes benzoic, gallic and syringic acid as well as the phenylpropenes caffeic and ferulic acid had only marginal effects on the K^+ evoked uptake of $^{45}Ca^{2+}$ (Table 1, **VI**). Methanol extract of the *P. emblica* L. leaves was tested for the possible interaction in the extracellular calicium transport due to the activity in human PMN functions. The extract did not possess any clear activity in the extracellular calcium fluxes (SUMMANEN, unpublished results).

$^{45}Ca^{2+}$ entry activators. Some of the flavonoids and simple phenolics appeared to be potent activators of calcium entry (see **Table 1**, **VI**). Isorhamnetin, morin and quercetin enhanced the K^+- evoked uptake of $^{45}Ca^{2+}$ by 52.4, 48.0 and 54.1 %, respectively at a concentration of 20 µg/ml. The flavonolglycoside quercitrin had a modest effect, while rutin and rhamnetin were without an effect on the calcium entry. Dose response studies were conducted for morin and quercetin using the concentrations 0.4, 4.0, 40.0 and 120 µg/ml. In these experiments, higher concentrations were used in order to see whether they would reverse the enhancing effect of these molecules to an inhibitor effect. However, even at the highest dose tested (120 µg/ml), both morin and quercetin still enhanced calcium entry (**Fig. 2**, **VI**).

These results led us to test whether the compounds would possess this kind of activity without prior K^+ depolarization.. The experiments were performed as earlier described in the absence of K^+ depolarization. The measurements showed that quercetin was able to increase the Ca^{2+} entry by 43.0 % also in the absence of depolarization. Both isorhamnetin and morin increased calcium entry by 13.0 % under these conditions. A possible mechanism mediating the isorhamentin, morin and quercetin effects on calcium entry could be *via* cellular cAMP, as quercetin has been previously shown to increase cAMP in human platelets, and prevent their aggregation (LANZA et al. 1987). To test this hypothesis the effects of quercetin on cAMP levels in GH_4C_1 was measured. Quercetin induced an increase in cAMP at different concentrations to increase $^{45}Ca^{2+}$ entry (**Fig. 3A**, **VI**). Furthermore, the PKA inhibitor H-89 abolished the quercetin-induced increase in $^{45}Ca^{2+}$ entry (**Fig. 3B**, **VI**), strengthening the conclusion that the enhanced entry of $^{45}Ca^{2+}$ evoked by quercetin is the result of an increase in cAMP and concomitant activation of PKA leading to upmodulation of the VOCCs.

The effect of quercetin and octyl gallate on VOCCs mediated membrane currents. Quercetin (20 µg/ml) induced a marked potentiation in the maximal Ca^{2+} currents observed following depolarisation from a holding potential of -70 mV to -10 - +10 mV (see **Fig. 4B**, **IV**). It also induced a slight shift in the current-voltage relationship of the VOCCs. The mean quercetin-induced increase in the transient current observed in experiments similar to that in **Fig. 4C** and **4D** (see **VI**), was 143.1 ± 4.2 %, and that of the late, delayed current 198.8 ± 10.0 % (n=5). The onset of the quercetin effect was fairly rapid, peaking in about 10 seconds after the onset of quercetin perfusion. Despite of the pronounced rundown of Ca^{2+} currents typical to the experimental conditions used here (TITIEVSKY et al. 1998), the effect of quercetin was always at least partially reversible (see **VI**, **Fig. 4B**). Octyl gallate had a very potent inhibitory effect on the $^{45}Ca^{2+}$ entry. Octyl gallate (20 µg/ml) produced almost complete inhibition of the VOCCs (-73.8 ± 12.2 %, values are the mean of all measurements of the current increase, n=4, see **VI**, **Fig. 5**), but displayed a clearly slower onset of action compared with quercetin.

The present study provides evidence that flavone has similar inhibitory activity to other classes of natural CCAs, *i.e.* furanocoumarins (VUORELA 1988), and that this activity is also comparable to that of verapamil hydrochloride. Rather surprisingly, the flavonols appeared to be potent activators of calcium entry. This finding does not support the theory that structurally related flavonoids would act in the same way. REVUELTA et al. (1997) have recently suggested that genistein, kämpherol, and quercetin possess a calcium antagonistic action in rat uterine smooth muscle but show a different behaviour toward the calcium channel blocker (CCBs) and the cellular cyclic AMP enhancer forskolin. Interestingly, quercetin has been

reported to inhibit partly purified protein kinase A (PKA) from rat brain (END et al. 1987). Other studies have shown that quercetin increases cellular cAMP (LANZA et al. 1987, DUARTE et al. 1993), and our results collaborate these findings. Our voltage clamp experiments show that quercetin markedly enhances both the transient and delayed Ca^{2+} currents, indicating that quercetin may affect both the T- and L-type VOCCs. Although further experiments are needed to elucidate the mechanism of quercetin-induced VOCC enhancement, the rapid onset and reversibility of quercetin indicate that it may have a direct action on the VOCCs.

6. Conclusions

Capillary electrophoresis (CE) can be used as a fast and rapid analytical technique for semi-identification of the constituents extracted from medicinal plant material. The reliability of the identification in micellar electrokinetic capillary chromatography (MEKC) can be clearly improved by using the two-marker technique and migration indices. The problems caused by the complexity of the mixture and the presence of several compounds in very low concentration can be overcome by combination of interpretive optimization methods. The present study showed that systematic optimization of an instrumented TLC method *i.e.* Automated Multiple Development (AMD) can lead to reliable and automatically repeatable analysis of complex mixtures. The densitometer scanning technique and video documentation system in TLC detection are equally suitable for making documents of TLC layers. However, video technology proved to enhance the value of TLC as a rapid and accurate method for separation and analysis using *Ph. emblica* L. (Euphorbiaceae) as a model plant. High speed quantification by video imaging from UV down to IR might be possible in the future, thus providing more information about the structure of compounds in multicomponent mixtures. For the first time the computerized "PRISMA" system was used in combination with the desirability function technique in designing an eluent mixture for the NP- and RP-TLC separation. The software method can thus be utilized in optimizing the solvent system for the separation of unknown compounds responsible for the biological activity of *P. emblica* leaves.

The present study on anti-inflammatory activity of the *P. emblica* suggests that the plant leaves contain as yet unidentified polar compound(s) with potent inhibitory activity on polymorphonuclear leukocytes (PMNs), as well as chemically different apolar molecule(s) that inhibit both prostanoid and leukotriene synthesis *e.g.* phospholipase A_2 activation. These findings are in line with the therapeutic actions of this plant in the management of inflammatory disorders in traditional medicine. Chemically and biologically well known phenolic compounds occuring in *P. emblica i.e.* ellagic acid, gallic acid, and rutin could not explain these actions. The unknown compounds present in *P. emblica* might have an important effect on PMN activation cascade by interacting with intracellular calcium release or Ca^{2+} influx into the PMNs. The calcium antagonistic activity studies showed that simple aromatic compounds and flavonoids have different effects on calcium dynamics which appear to be attributed to direct actions on the VOCCs in clonal rat pituitary GH_4C_1 cells.

References

ACKMAN, R.G., MACLEOD, C.A. and BANERJEE, A.K. (1990): An overview of analyses by Chromarod-Iatroscan TLC-FID. *J. Planar Chromatogr.* **3:** 450–490.

ALCARAZ, M.J. and FERRANDIZ, M.L. (1987): Modification of arachidonic metabolism by flavonoids. *J. Ethnopharmacol.* **21:** 209–229.

ALDSTADT, J.H. and DEWALD, H.D. (1992): Determination of heavy metals by thin-layer chromatography-square-wave anodic stripping voltametry. *Anal. Chem.* **64:** 3176–3179.

ANDERSSON, J. and VAN NIEKIRK, P.J. (1987): High-performance liquid chromatographic determination of antioxidants in fats and oils. *J. Chromatogr.* **394:** 400–402.

ANDRIKOPOULOS, N.K., BRUESCHWEILER, H., WELBER, H. and TAESCHLER, C. (1991): HPLC analysis of phenolic antioxidants, tocopherols and triglycerides. *J. Am. Oil. Chem. Soc.* **68:** 359–364.

ANNAPURANI, T.K. (1989): High performace liquid chromatographic analysis of certain phenols and phenolic acids from plants. *Curr. Sci.* **58:** 190–191.

ARUOMA, O.I., MURCIA, A., BUTLER, J. and HALLIWELL (1993): Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem.* **41:** 1880–1885.

ASMAWI, M.Z., KANKAANRANTA, H., MOILANEN, E. and VAPAATALO, H. (1992): Anti-inflammatory activities of *Emblica officinalis* GAERTN leaf extracts. *J. Pharm. Pharmacol.* **45:** 581–584.

AUSTIN, R.E. and WYATT, D.M. (1980): Rapid method for analysis of refined vegetable oils for TBHQ by gas chromatography. *J. Am. Oil Chem. Soc.* **57:** 422–424.

BAGGIOLINI, M. BOULAY, F., BADWEY, J.A. and CURNUTTE, J.T. (1993): Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burts. *FASEB J.* **7:** 1004–1010.

BASA, S.C. and SHRINIVASULU, C. (1987): Constituents of leaves of *Phyllanthus emblica* Linn. *Indian J. Nat. Prod.* **3:** 13–14.

BASCANDS, J.L. PECHER, C., ROUNAUD, S., EMOND, C., TACK, J.L., BASTIE, M.J., BURCH, R., REGOLI, D. and GIROLAMI, J.P. (1993): Evidence for existence of two distinct bradykinin receptors on rat mesengial cells. *Am. J. Physiol.* **264:** F548–F556.

BAYNE, C.K. and MA, C.Y. (1987): Optimization of solvent composition for high performance thin-layer chromatography. *J. Liq. Chromatogr.* **10:** 3529.

BEALE, S. C. (1998): Capillary electrophoresis. *Anal. Chem.* **70:** 279R-300R.

BEESLEY, T.E. (1985): Current instrumentation for thin layer chromatography. *J. Chrom. Sci.* **24:** 525–531.

BERRIDGE, M.J. (1993): Inositol trisphosphate and calcium signalling. *Nature* **361:** 315–325.

BIERER, D., DENER, J.M., DUBENKO, L. GERBER, E., LITVAK, J., PETERLI, S., PETERLI-ROTH, P., TRUONG, TV. MAO, G. and BAUER, B.E. (1995): Novel 1,2-dothiins: synthesis, molecular modeling studies, and antifungal activity. *J. Med. Chem.* **38:** 2628–2648.

BJERGEGAARD, C., MICHAELSEN, S. and SORENSEN, H. (1992): Determination of phenolic carboxylic acids by micellar electrokinetic capillary chromatography and evaluation of factors affecting the method. *J. Chromatogr.* **608:** 403–411.

BOKOCH, G.M. (1993): Biology of Rap proteins, members of the ras superfamily of GTB-binding proteins. *Biochem. J.* **289:** 17–24.

BORGEAT, P. SAMUELSSON, B. (1979): Arachidonic acid metabolism in polymorphonuclear leukocytes: effect of ionophore A 23187. *Proc. Natl. Acad. Sci.* **76:** 2148–2152.

BRUHN, J.G. and HOLMSTEDT, B. (1981): In: Natural products as medicinal agents. Beal, J. and Reinhard, E., Eds., Hippokrates, Stuttgart.

BURGER, K.D. AND TENGLER, H. (1986): Automated Multiple Development—A new technique of thin layer chromatography, pp. 193-205. In: Planar Chromatography Vol. I. Kaiser, R.E., Ed. Dr. Alfred Hüthig Verlag, Heidelberg–Basel–New York.

BURKILL, I. H. (1966): A Dictionary of the Economic Products of the Malay Peninsula, Vol. 1, Ministry of Agriculture and Co-operatives, Kuala Lumpur.

BUSCH, K.L. (1992): Coupling thin layer chromatography with mass spectrometry. *J. Planar Chromatogr.* **5:** 72–79.

CAHALAN and NEHER (1992): Patch clamp techniques: An Overview. *Methods Enzymol.* **207:** 3–14.

CALIXTO, J.B., SANTOS, A.R.S., FILHO, V.C. and TUNES, R.A. (1998): A review of the plants of the genus *Phyllanthus*: Their chemistry, pharmacology, and therapeutic potential. *J. Med. Biol. Res.* **31:** 225–258.

CARTER, S.K. and LIVINGSTON, R.B. (1976): Plant products in cancer chemotherapy. *Cancer Treat. Rep.* **60:** 1141–1156.

CHANG R.L., HUANG M.T., WOOD A.W., WONG C.Q., NEWMARK H.L., YAGI H., SAYER J.M., AND CONNEY A.H. (1985): Effect of ellagic acid and hydroxylated flavonoids on the tumourigenicity of benzo(a)pyrene and (+)-7ß, 8alfa-dihydro-9alfa,10alfa-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene on mouse skin in newborn mouse. *Carcinogenesis* **6:** 1127–1133.

CHARLIER, C., BRUYNEEL, E., LECHANTEUR, C., BRACKE, M., MARCEL, M. and CATRONOVO, V. (1996): Enhancement of tamoxifen-induced E-cadherin function by Ca^{2+} channel antagonists in human breast cancer MCF7/6 cells. *Eur. J. Pharmacol.* **317:** 413–416.

CIMPOIU, C., JANTSCHI, L. and HODISAN, T. (1998): A new method for mobile phase optimization in high-performance thin-layer chromatography (HPTLC). *J. Planar Chromatogr.* **11:** 191–194.

CLAPHAM, D.E. (1995): Calcium signalling. *Cell* **80:** 259–268.

CORDELL, G.A. (1995): Changing strategies in natural product chemistry. *Phytochemistry* **40:** 1585–1612.

CSERHÁTI, T. and FORGÁCS, E. (1997): Trends in thin-layer chromatography. *J. Chromatogr. Sci.* **35:** 383–391.

CSERHÁTI, T. and FORGÁCS, E. (1998): Hyphenated techniques in thin-layer chromatography. *J. AOAC Int.* **81(2):** 329–332.

DALLENBACH-TÖLKE, K. NYIREDY, Sz., MEIER, B. and STICHER, O. (1986): Optimization of overpressured layer chromatography of polar, naturally occuring compounds by the " PRISMA" model. *J. Chromatogr.* **365:** 63–72.

DAMORADAN, M. and SHRINIVASAN, M. (1935): Vitamin C content of some Indian Plant materials. *Curr. Sci.* **3:** 553.

DEMING, S.N. (1991): Multiple-criteria optimization. *J. Chromatogr.* **550:** 15–25.

DEMING, S.N., PALASOTA, J.M., LEE, J. and SUN, L. (1989): Computer-assisted optimization in high-performance liquid chromatographic method development. J. *Chromatogr.* **485:** 15–25.

DESAI, H.K., GAWAD, D.H., JOSHI, B.S., PARTHASATHY, P.C., RAVINDRANATH, K.R., SAINDANE, M.T., SIDHAVE, A.R. and VISWANATHAN, N. (1977): Chemical investigation of Indian plants: Part X. *Indian J. Chem.* **15B 3:** 291–293.

DHIR, H., AGARWAL, K. and SHARMA, A. and TALUKDER, G. (1991): Modifying role of *Phyllanthus emblica* and ascorbic acid against nickel clastogenicity in mice. *Cancer Lett.* **59:** 9–18.

DHIR, H., ROY, A.K., SHARMA, A. and TALUKDER , G. (1990): Protection afforded by aqueous extracts of *Phyllanthus* species against cytotoxicity induced by lead and aluminium salts. *Phytoter. Res.* **4:** 172–176.

DIXON, R.A. and PAIVA, N.L. (1995): Stress-induced phenylpropanoid metabolism. *Plant-Cell* **7:** 1085–1097.

DUARTE, J., PÉREZ-VIZCAINO, F., ZARZUELO, JIMÉNEZ, J. and TAMARGO, J. (1993): Vasodilator effects of quercetin in isolated rat vascular smooth muscle. *Eur. J. Pharmacol.* **239:** 1–7.

EBEL, S. (1996): Quantitative analysis in TLC and HPTLC. *J. Planar Chromatogr.* **9:** 4–15.

EBEL, S., BIGALKE, H-J. and VÖLKL, S. (1987): Proceedings of the IVth International Symposium On Instrumental HPTLC (Selvino), p. 113, In: Traitler, H., Studer, A. and Kaiser, R.E. Eds., Institut für Chromatographie, Bad Dürkheim.

EBEL, S. and VÖLKL, S. (1990): DC-Analytik von Naturstoffen. Verbesserung mit automatisierter Mehrfachentwicklung (AMD). *DAZ* **130:** 2162–2169.

EDWARDS, S.W. (1994): Biochemistry and physiology of the neutrophil. Cambridge University Press, New York.

EL-MEKKAWY, S., MESELHYM.R., KUSUMOTO, I.T., KADOTA, S. HATTORI, M. and NAMBA T. (1995): Inhibitory effects of Egyptian folk medicines on human immunodefience virus (HIV) reverse transcriptase. *Chem. Pharm. Bull.* **43:** 641–648.

END, D., LOOK, R., SHAFFER, N., BALLES, E. and PERSICO, F. (1987): Non-selective inhibition of mammalian protein kinases by flavonoids *in vitro*. *Res. Commun. Chem. Pathol. Pharmacol.* **56:** 75–86.

ERESHEFSKY, L., RIESENMAN, C., TRUE, J.E. and JAVORS, M. (1996): Serotonin-mediated increase in cytosolic [Ca^{++}] in platelets of risperidone-treated schizophrenia patients. *Psychopharmacol. Bull.* **32:** 101–106.

EVERALL, N.J., CHALMERS, M. and NEWTON, I.D. (1991): *In situ* identification of thin-layer chromatography fractions by Fourier-transform Raman spectroscopy. *Appl. Spectr.* **46:** 597–601.

FARNSWORTH, N. (1988): Screening plants for new medicines, pp. 83-97. In: Biodiversity. Wilson, E-O., Ed., National Academy Press, Washington.

FARNSWORTH, N.R., AKERELE, O., BINGEL, A.S., SOEJARTO, D.D. and GUO, Z. (1985): Medicinal plants in therapy. *Bull. WHO* **63:** 965–981.

FERES, T., PAIVA, A.C.M., PAIVA, T.B. (1992): BK_1 and BK_2 bradykinin receptors in the rat duodenum smooth muscle. *Br. J. Pharmacol.* **107:** 991–995.

FORMICA, J.V. and REGELSON W. (1995): Review of the biology of quercetin and related bioflavonoids. *Fd. Chem. Toxic.* **33:** 1061–1080.

FRIED, B. and SHERMA, J. (1994): Thin-layer chromatography: techniques and applications. Handbook of thin-layer chromatography, Marcel Dekker, Inc., New York-Basel-Hong Kong.

FUJIWARA, S. and HONDA, S. (1986): Determination of cinnamic acid and its analogues by electrophoresis in a fused silica capillary tube. *Anal. Chem.* **58:** 1811–1814.

FUNK, C.D., FUNK, L.B., KENNEDY, M.E., PONG, A.S., FITZGERALD, G.A. (1991): Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.* **5:** 2304–2312.

GEISS, F. (1987): Fundamentals of thin layer chromatography (Planar chromatography). Dr. Alfred Hüthig Verlag, Heidelberg-Basel-New York.

GHOSAL, S. TRIPATHI, V.K. and CHAUHAN, S. (1996): Active constituents of *Emblica officinalis*: Part I. The chemistry and antioxidant activity of two new hydrolysable tannins, emblicanin A and B. *Indian J. Chem.* **35B 9:** 941–948.

GIRI,A.K. and BANERJEE, T.S. (1986): Antagonistic activity of herbal drug (*Phyllanthus emblica*) on cytological effects of environmental chemicals on mammalian cells. *Cytologia* **51:** 375–380.

GLAJCH, J.L., KIRKLAND, J.J., SQUIRE, K.M. and MINOR, J.M. (1980): Optimization of solvent strength and selectivity for reversed-phase liquid chromatography using an interactive mixture-design statistical technique. *J. Chromatogr.* **199:** 57–79.

GLAJCH, J.L. and SNYDER, L.R. (1990): Computer-assisted method development for high-performance liquid chromatography. Elsevier, Amsterdam.

GOCAN, S., GIMPAN, G. and MURESAN, L. (1996): Automated multiple development thin layer chromatography of some plant extracts. *J. Pharm. Biomed. Anal.* **14:** 1221–1227.

GOLGAN, S.T. and POLLARD, E.B. (1992): Computer-assisted separations of 5-chlorooxindole and potential process-related impurities. *LC-GC Int.* **5:** 31–33.

GONG, Y., BLOK, L.J., PERRY, J.E., LINDZEY, J.K. and TINDALL, D.J. (1995): Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* **136:** 2172–2178.

GRINBERG, N. (1990): Relationship between thin-layer and column chromatography. *Chromatogr. Sci. (Mod. Thin-Layer Chromatogr.)* **52:** 453–463.

GULATI,, R.K., AGARWAL, S. and AGRAWAL, S.S. (1995): Hepatoprotective studies on *Phyllanthus emblica* Linn. and quercetin. *Ind. J. Exp. Biol.* **33:** 261–268, erratum **33:** preceding table of contents.

HARBORNE, J.B., Ed. (1994): The flavonoids—advances in research since 1986. Chapman and Hall, London.

HASLAM, E. (1998): Practical polyphenolics: from structure to molecular recognition and physiological action. Cambridge University Press, New York, NY.

HERRMANN, R., LINDEMANN, A., GAUSS, J. and MERTELSMANN, R. (1990): Cytokine-stimulation of prostaglandin synthesis from endogenous and exogenous arachidonic acids in polymorphonuclear leukocytes involving activation and new synthesis of cyclooxygenase. *Eur. J. Immunol.* **20:** 2513–2516.

HO, C.T. (1992a): Phenolic compounds in food. An overview, pp. 2-7. In: ACS Symposium series 507: Phenolic Compounds in Food and Their Effects on Health II. Antioxidants and Cancer Prevention. Ho C.T., Lee D.Y., Huang M-T. Eds., ACS, Washington, DC.

HO, C.T. (1992b): Phenolic compounds in food. An overview, pp. 8-34. In: ACS Symposium series 507: Phenolic Compounds in Food and Their Effects on Health II. Antioxidants and Cancer Prevention. Ho C.T., Lee D.Y., Huang M-T. Eds., ACS, Washington, DC.

HOF, R. and VUORELA, H. (1983): Assessing calcium antagonism on vascular smooth muscle: a comparison of three methods. *J. Pharmacol. Meth.* **9:** 41–52.

HOFFSTETTER-KUHN, S., PAULUS, A., GASSMANN, E. and WIDMER, H. (1991): Influence of borate complexation on the electrophoretic behaviour of carbohydrates in capillary electrophoresis. *Anal. Chem.* **63:** 1541–1547.

HOSTETTMANN, K., TERREAUX, C. MARSTON, A. and POTTERAT, O. (1997): The role of planar chromatography in rapid screening and isolation of bioactive compounds from medicinal plants. *J. Planar Chromatogr.* **10:** 251–257.

HUI, B.W. and SUNG, M.L. (1968): An examination of the Euphorbiaceae of Hong Kong. II. The occurence of epitaraxenol and other triterpenoids. *Aust. J. Chem.* **21:** 2137–2140.

HWANG, K.T. and MAERKER, G. (1993): Quantitation of cholesterol oxidation products in unirriadiated and irradiated meats. *J. Am. Oil. Chem. Soc.* **70:** 371–375.

HWANG, S.B., CHANG, M.N. and GARCIA, M.L. (1987): L-652, 469—a dual receptor antagonist of platelet activating factor and dihydropyridines from *Tussilago farfara* L. *Eur. J. Pharmacol.* **141:** 269–281.

HÄRMÄLÄ, P. (1991): Study on the isolation and chromatographic behaviour of coumarins from Angelica (*Angelica archangelica*) roots, thesis, 58 p., University of Helsinki, J-Paino Ky, Helsinki.

HÄRMÄLÄ, P., VUORELA, H., NYIREDY Sz., KALTIA, S., STICHER, O. and HILTUNEN, R. (1992): Strategy for the isolation and identification of coumarins with calcium antagonistic properties from the roots of *Angelica archangelica*. *Phytochem. Anal.* 3: 42–48.

IBRAHIM, R. and BARRON, D. (1989): Phenylpropanoids, pp. 73-111. In: Methods in plant biochemistry: Plant Phenolics. Harborne, J., Ed., Academic Press, London.

ICHIKAWA, K., KINOSHITA, T., NISHIBE, S. and SANKAWA U. (1986): The Ca^{2+} activity of lignans. *Chem. Pharm. Bull.* **34:** 3514–3517.

ICHIKAWA, K., KINOSHITA, T. and SANKAWA U. (1989): The screening of Chinese crude drugs for Ca^{2+} antagonistic activity: identification of active principles from the aerial part of *Pogostemon cablin* and the fruits of *Prunus mume*. *Chem. Pharm. Bull.* **37:** 345–348.

IHANTOLA-VORMISTO, A., SUMMANEN, J., KANKAANRANTA, H., VUORELA, H., ASMAWI, M.Z. and MOILANEN, E. (1997): Anti-inflammatory activity of extracts from leaves of *Phyllanthus emblica*. *Planta Med.* **63:** 518–524.

ISHII, D. and TAKEUCHI, T. (1989): Unified fluid chromatography. *J. Chromatogr. Sci.* **27:** 71–76.

ISSAQ, H.J. and SEBURN, K.E. (1989): Graphic presentation of binary mobile phase optimization in thin-layer chromatography. *J. Liq. Chromatogr.* **12:** 3121–3128.

JACOB, A, PANDEY, M., KAPOOR, S. and SAROJA, R. (1988): Effect of the Indian gooseberry (amla) onserum cholesterol levels in men aged 35-55 years. *Eur. J. Clin. Nutr.* **42:** 939–944.

JAENCHEN, D. (1996a): Planar chromatography (Instrumental thin-layer chromatography), pp.129-148. Handbook of thin-layer chromatography. Sherma, J. and Fried, B. Eds. Marcel Dekker, New York, NY.

JAENCHEN, D. (1996b): CBS, Camag bibliography service, September 1996, No. 77, Camag Scientific Inc., Wilmington, NC.

JANTAN, I.B., KANG, Y.H., SUH, D.Y. and HAN, B.H. (1996): Inhibitory effects of Malaysian medicinal plants on the platelet-activating factor (PAF) receptor binding. *Nat. Prod. Sci.* **2:** 86–89.

JOFFE, R.T., LEVITT, A.J. and YOUNG, L.T. (1996): The thyroid, magnesium and calcium in major depression. *Biol. Psychiatry* **40:** 428–429.

JORK, H., FUNK, W., FISCHER, W. and WIMMER, H. (1990): Thin-layer chromatography: reagents and detection methods Vol. 1a. VCH Verlagsgesellschaft mbH, Weinheim.

JOSE, J.K., JOY, K.L. and KUTTAN, R. (1999): Effect of *Emblica officinalis, Phyllanthus amarus* and *Picrorrhiza kurroa* on *N*-nitrosodiethylamine induced hepatocarcinogenesis. *Cancer Lett.* **136:** 11–16.

JOSE, J.K. and KUTTAN, R. (1995): Antioxidant activity of *Emblica officinalis*. *J. Clin Biochem. Nutr.* **19:** 63–70.

JOSE, J.K., KUTTAN, G. GEORGE, J. and KUTTAN, R. (1997): Antimutagenic and anticarcinogenic activity of *Emblica officinalis* GAERTN. *J. Clin. Biochem. Nutr.* **22:** 171–176.

JUMPPANEN, J.H. and RIEKKOLA, M-L. (1995): Marker techniques for high accuracy identification in CZE. *Anal. Chem.* **67:** 1060–66.

JUMPPANEN, J.H., SIRÉN, H., RIEKKOLA, M-L., SÖDERMAN, O. (1993): Correlation of resolution with fractional coefficients and pK, values in capillary electrophoresis of four diuretics: determination of electric field strength and electroosmosis velocity. *J. Microcol. Sep.* **5:** 451–457.

JUNIOR, A.A.S. and VIZZOTTO (1996): ref. in: CALIXTO, J.B., SANTOS, A.R.S., FILHO, V.C. and TUNES, R.A. (1998): A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. *J. Med. Biol. Res.* **31:** 225–258.

KAISER, R. (1969): Thin-layer chromatography in direct coupling with gas chromatography and mass spectrometry. *Chem Britain* **5:** 54–61.

KALÁSZ, H. and BATHORI, M. (1997): Present status and future perspectives of thin-layer chromatography. *LC-GC Int.* **10:** 440–445.

KANKAANRANTA, H. (1995): Prostanoid-independent effects of fenamates on polymorphonuclear leukocytes, thesis 84 p., University of Tampere, Vammalan Kirjapaino Oy, Vammala.

KAPIOTIS, S., HERMANN, M., HELD, I., SEELOS, C., EHRINGER, H. and GMEINER, B.M.K. (1997): Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. *Atheroscler. Thromb. Vasc. Biol*. **17:** 1868–2874.

KATOPODIS, J.N., KOLODNY, L., JY, W., HORSTMAN, L.L. DE MARCHENA E.J., TAO, J.G., HAYNES, D.H. and AHN, Y.S. (1997): Platelet microparticles and calcium homeostasis in acute coronary ischemias. *Am. J. Hematol.* **54:** 95–101.

KATZ, A.M. (1997): Molecular biology of calcium channels in the cardiovascular system. *Am. J. Cardiol.* 80: 117–122.

KHALEDI, M.G., SMITH, S.C. and STRASTERS, J.K. (1991): Micellar electrokinetic capillary chromatography in acidic solutes: migration behaviour and optimization strategies. *Anal. Chem.* **63:** 1820–1830.

KHANNA, P. and BANSAL, R. (1975): Phyllantidine & phyllantine from *Emblica officinalis* Gaertn leaves, fruits, & *in vitro* tissue cultures., *Indian J. Exp. Biol.* **13:** 82–83.

KLIN, M., SMOGOREWSKI, M. and MASSRY, S.G. (1995): Chronic renal failure increases cytosolic Ca^{2+} of hepatocytes. *Am. J. Physiol.* **269:** G103–G109.

KOCH, B.D., FAUROT, G.F., KOPANITSA, M.V. AND SWINNEY, D.C. (1994): Pharmacology of a Ca^{2+} influx pathway activated by emptying the intracellular Ca^{2+} stores in HL-60 cells: evidence that a cytochrome P-450 is not involved. *Biochem. J.* **302:** 187–190.

KONG, R.C. SACHOK, B. and DEMING S.N. (1980): Combined effects of pH and surface-active-ion concentration in reversed-phase liquid chromatography. *J. Chromatogr.* **199:** 307–316.

KOVAR, K.-A., ENSSLIN, H.K., FREY, O.R., RIENAS, S. and WOLFF, S.C. (1991): Identification and estimation of complex mixtures by direct-TLC-IR online coupling. *GIT Special Chromatographie* **11** 95–98, 100–101.

KROES, , B.H., VAN DEN BERG, J.J., QUARLES VAN UFFORD, H.C., VAN DIJK, H. and LABADIE R.P. (1992): Anti-inflammatory activity of gallic acid. *Planta Med.* **58:** 499–504.

KUMAR KC, S., and MÜLLER, K. (1999): Medicinal plants from Nepal; II. Evaluation as inhibitors of lipid peroxidation in biological membranes. *J. Ethnopharmacol.* **64:** 135–139.

KUSUMOTO, I.T., NAKABAYASHI, T., KIDA, H., MIYASHIRO, H., HATTORI, M., NAMBA, T. and SHIMOTOHNO, K. (1995): Screening of various plant extracts used in ayurvedic medicine for inhibitory effects on human immunodeficiency virus type 1 (HIV-1) protease. *Phytother. Res.* **9:** 180–184.

LANZA, F., BERETZ, A., STIERLE, A., CORRE, G. and CZANAVE, J. (1987): Cyclic nucleotide phosphodiesterase inhibitors prevent aggregation of human platelets by raising cyclic AMP and reducing cytoplasmic free calcium mobilization. *Thromb. Res.* **45:** 477–484.

LAUMAS, K.R. and SESHADRI, T.R. (1958): Chemical components of the bark of *Phyllanthus emblica*. *J. Sci. Ind. Res. (India)* **17B:** 167–168.

LAYMAN, L.R., TARGAN, D.A., GILMORE, D.A. and DENTON, M.B. (1995): Photodocumentation of thin layer chromatography plates. J. *Planar Chromatogr.* **8:** 397–400.

LEE, M., ALDRED, K., LEE, E. and FELDMAN, E. (1992): Aspirin-induced acute gastric mucosal injury is a neutrophil-dependent process in rats. *Am. J. Physiol.* **263:** G920–G926.

LESCA P. (1983): Protective effects of ellagic acid and other plant phenols on benzo(a)pyrene-induced neoplasia in mice. *Carcinogenesis*. **4:** 1651–1653.

LI, G.R., FERRIER, G.R. and HOWLETT, S.E. (1995): Calcium currents in ventricular myocytes of prehypertrophic cardiomyopathic hamsters. *Am. J. Physiol.* **268:** H999–1005.

LIANG, H.-R. (1997): Phytochemical and electrophoretic characterization and quantitative structure-migration relationships of flavonoids from Epimedium, thesis, 55 p., University of Helsinki, Cosmoprint, Helsinki.

LIANG H.-R., VUORELA, P., VUORELA, H., RIEKKOLA, M.-L. and HILTUNEN, R. (1998): The statistical evaluation of migration parameters of flavonoids in capillary electrophoresis with reference to structural descriptors. *J. Liq. Chrom. & Rel. Technol.* **21:** 625–643.

LIU, K.C.S.C., LIN, M.T., LEE, S.S., CHIOU, J.F.REN, S.J. and LIEN, E.J. (1999): Antiviral tannins from two *Phyllanthus* species. *Planta Med.* **65:** 43–46.

LODI, G., BETTI, A., MENZIANI, E., BRANDOLINI, V. and TOSI, B. (1991): Some examples of automated multiple development (AMD) gradient optimization. *J. Planar Chromatogr.* **4:** 106–110.

VAN LUIJTELAAR, E.L., ATES, N. and COENEN, A.M. (1995): Role of L-type calcium channel modulation in nonconvulsive epilepsy in rats. *Epilepsia* **36:** 86–92.

MACHEIX, J.J. and FLEURIET, A. (1998): Phenolic acids in fruits, pp. 35-59. In: Flavonoids in health and disease. Rice-Evans, C.A. and Packer, L. Eds. Marcel Dekker Inc., New York.

MALL, T. (1998): TLC-evaluation: densitometry or video documentation. In: Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, pp. 103-109. Visegrad, Hungary, 16–19.05.1998.

MAND, J.K., SONI, G.L., GUPTA, P.P. and SINGH, R. (1991): Effect of amla (*Emblica officinalis*) on the development of atherosclerosis on hypercholesterolemic rabbits. *J. Res. Edu. Ind. Med.* **10:** 1–7.

MARKHAM, K.R. and BLOOR, S.J. (1998): Analysis and identification of flavonoids in practice. In: Flavonoids in health and disease, pp. 1-33. Rice-Evans, C.A. and Packer, L. Eds. (1998), Marcel Dekker Inc., New York.

MARKOWSKI, W. (1993): Computer-aided optimization of gradient multiple development thin-layer chromatography. Part II. Multi-stage development. *J. Chromatogr.* **635:** 283–289.

MARKOWSKI, W. (1996): Computer-aided optimization of gradient multiple development thin-layer chromatography. III. Multistage development over a constant distance. *J. Chromatogr. A* **726:** 185–192.

MARTIN, M.L. DIAZ, M.T., MONTERO, M.J., PRIETO, P. ROMAN, L. SAN and CORTES, D. (1993): Antispasmodic activity of benzylisoquinoline alkaloids analogous to papaverine. *Planta Med.* **59:** 63–67.

MATHUR, R., SHARMA, A., DIXIT, V.P. and VARMA, M. (1996): Hypolipidaemic effect of fruit juice of *Emblica officinalis* in cholesterol-fed rabbits. *J. Ethnopharmacol.* **50:** 61–68.

MATYSIK, G. (1996): Modified programmed multiple gradient development (MGD) in the analysis of complex plant extracts. *Chromatographia* **43:** 39–43.

MATYSIK, G. and GIRYN, H. (1996): Gradient thin-layer chromatography and densitometry determination of *Alternaria* mycotoxins. *Chromatographia* **42:** 555–558.

MCPHAIL, L.C., QUALLIOTINE-MANN, D., AGWU, D.E. and MACCALL C.E. (1993): Phospholipases and activation route of the NADPH oxidase. *Eur. J. Haematol.* **51:** 294–300.

MELTZER, H.M. and MALTERUD, K.E. (1997): Can dietary flavonoids influence the development of coronary heart disease. *Scand. J. Nutr.* **41:** 50–57.

MENZIANI, E., TOSI, B., BONORA, A., RESCHIGLIAN, P. and LODI, G. (1990): Automated multiple development high-performance thin-layer chromatographic analysis of natural phenolic compounds. *J. Chromatogr.* **511:** 396–401.

MIDDLETON, E., Jr. AND KANDASWAMI, C. (1992): Effects of flavonoids on immune and inflammatory cell functions. *Biochem. Pharmacol.* **43:** 1167–1179.

MIDDLETON, E., Jr. AND KANDASWAMI, C. (1994): The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer, pp. 619-652. In: The flavonoids—advances in research since 1986, Harborne, J, Ed. Chapman and Hall, London.

MINCSOVICS, E. and TYIHÁK, E. (1988): Forced-flow planar liquid chromatographic techniques. *J. Planar Chromatogr.* **1:** 6–19.

MORALES, M.A. and LOZOYA, X. (1994): Calcium-antagonistic effects of quercetin on aortic smooth muscle. *Planta Med.* **60:** 313–317.

MORGAN, S.L.and JACQUES, C.A. (1978): Response surface evaluation and optimization in gas chromatography. *J. Chromatogr. Sci.* **16:** 500–505.

MORIN, P. and DREUX, M. (1993): Factors influencing the separation of ionic and non-ionic chemical natural compounds in plant extracts by capillary electrophoresis. *J. Liq. Chromatogr.* **16:** 3735–3755.

MUKHTAR, H., DAS, M., KHAN, W.A., WANG, Z.Y., BLIK, D.P., and BICKERS, D.R. (1988) Exceptional activity of tannic acid among naturally occuring plant phenols in protecting against 7,12-dimethylbenz(a)anthracene-, benzo(a)pyrene-, 3-methylcholantrene- and N-methyl-N-nitrosourea-induced skin tumourigenesis in mice. *Cancer Res.* **48:** 2361–2365.

MURRAY, T.M. (1996): Calcium nutrition and osteoporosis. *Can. Med. Assoc. J.* **155:** 935–939.

NAGY-TURÁK, A., VÉGH, Z. and FERENCZI-FODOR, K. (1995): Validation of the quantitative planar chromatographic analysis of drug substances III. Robustness testing in OPLC. *J. Planar Chromatogr.* **8:** 188–193.

NANDI, P., TALUKDER, G. and SHARMA, A. (1997): Dietary chemoprevention of clastogenic effects of 3,4-benzo(a)pyrene by *Emblica officinalis* GAERTN fruit extract. *Brit. J. Cancer* **76:** 1279–1283.

NEUHAUS-CARLISLE, K., VIERLING, W. and WAGNER, H. (1993): Calcium-channel blocking activity of essential oils from *Petroselinum crip.*, *Apium graveolens* and isolated phenylpropane constituents. *Pharm. Pharmacol. Lett.* **3:** 77–79.

NEUHAUS-CARLISLE, K., VIERLING, W. and WAGNER, H. (1997): Screening of plant extracts and plant constituents for calcium-channel blocking activity. *Phytomedicine* **4:** 67–71.

NI, Z., SMOGOREWSKI, M. and MASSRY, S.G. (1995): Elevated cytosolic calcium of adipocytes in chronic renal failure. *Kidney Int.* **47:** 1624–1629.

NIE, L., OISHI, Y., DOI, I., SHIBATA, H. and KOJIMA, I. (1997): Inhibition of proliferation of MCF-7 breast cancer cells by a blocker of Ca(2+)-permeable channel. *Cell Calcium* **22:** 75–82.

NIZZAMUDDIN, M.D., HOFFMAN, J. and LARM, O. (1982): Fractionation and characterization of carbohydrates from *Emblica officinalis* GAERTN fruit. *Swedish J. Agric. Res.* **12:** 3–7.

NOREEN, Y. (1997): Separation and bioassay in identification of cyclooxygenase inhibitors from plants, with emphasis on flavonoids, thesis, 55 p., Comprehensive summaries of Uppsala dissertations from the Faculty of Pharmacy 173, Uppsala.

NYIREDY, Sz. (1987): Application of the “PRISMA” model for the selection of eluent-systems in Over Pressure Layer Chromatography (OPLC), Labor MIM, Hungary.

NYIREDY, Sz., DALLENBACH-TÖLKE, K. and STICHER, O. (1988): The “PRISMA” optimization system in planar chromatography. *J. Planar Chromatogr.* **1:** 336–342.

NYIREDY, Sz., DALLENBACH-TÖLKE, K. and STICHER, O. (1989): Correlation and prediction of the k´ values for mobile phase optimization in HPLC. *J. Liq. Chromatogr.* **12:** 95–116.

NYIREDY, Sz., ERDELMEIER, C.A.J., DALLENBACH-TÖLKE, K., NYIREDY-MIKITA, K. and STICHER, O. (1986): Preparative on-line overpressured layer chromatography (OPLC): A new separation technique for natural products. *J. Nat. Prod.* **49:** 885–891.

NYIREDY, Sz., ERDELMEIER, C.A.J., MEIER, B. and STICHER, O. (1985a): “PRISMA” - Optimierung der mobilen Phase in der präparativen CLC. *Chromatographie Suppl.* **4:** 24–30.

NYIREDY, Sz., ERDELMEIER, C.A.J., MEIER, B. and STICHER, O. (1985b): “PRISMA”: Ein Modell zur Optimierung der mobilen Phase für die Dünnschichtchromatographie, vorgestellt anhand verschiedener Naturstofftrennungen. *Planta Med.* **51:** 241–246.

NYIREDY, Sz., MEIER, B., ERDELMEIER, C.A.J. and STICHER, O. (1985c): “PRISMA”: A geometrical design for solvent optimization in HPLC. *HRC & CC* **8:** 186–188.

OMORI, T., KOMATA, Y. and TAKASHI, M. (1998): Let’s make our own automated developing chamber for TLC, pp. 3–8. In: Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, Visegrad, Hungary, 16–19.05.1998.

OTTO, M. and WEGSCHEIDER, W. (1983): Multifactor model in the optimization of selectivity in reversed-phase chromatography. *J. Chromatogr.* **258:** 11–22.

OUTINEN, K., HAARIO, H., VUORELA, P., NYMAN, M., UKKONEN, E. and VUORELA, H. (1998): Optimization of selectivity in HPLC using desirability functions and mixture designs according to "PRISMA". *Eur. J. Pharm. Sci.* **6:** 197–205.

OUTINEN, K., VUORELA, H. and HILTUNEN R. (1996): Optimization of HPLC separation by computer assisted optimization methods. Comparison of three methods. *Eur. J. Pharm. Sci.* **4:** 199–210.

OZAVA, S. and KIMURA, N. (1982): Calcium channel and prolactin release in rat clonal pituitary cells: effects of verapamil. *Am. J. Physiol.* **243:** E68–E73.

PAGE, B.D. and KENNEDY, B.P.C. (1976): Rapid determination of butylated hydroxyanisole, tertbutylhydroquinone, and propyl gallate in edible oils by electron capture gas-liquid chromatography. *J. Assoc. Off. Anal. Chem.* **59:** 1208–1212.

PELANDER, A., SIVONEN, K., OJANPERÄ, I. and VUORELA, H. (1997): Retardation behavior of cyanobacterial hepatotoxins in the irregular part of the “PRISMA” model for thin layer chromatography. *J. Planar Chromatogr.* **10:** 434–440.

PELANDER, A., SUMMANEN, J., YRJÖNEN, T., HAARIO, H., OJANPERÄ, I. and VUORELA H. (1999): Optimization of separation in TLC using desirability functions and mixture designs according to “PRISMA”. *J. Planar Chromatogr.* **12:** accepted for publication.

PERRY, L.M. (1980): Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses, pp. 149–150. MIT Press, Cambridge.

PETROVIC, M., KASTELAN-MACAN, M., LAZARIC, K. and BABIC S. (1999): Validation of thin-layer chromatography quantitation determination with CCD camera and slit-scanning densitometer. *J. AOAC Int.* **82:** 25–30.

PEZZUTO, J.M. (1997): Plant-derived anticancer agents. *Biochem. Pharmacol.* **53:** 121–133.

PILLAY, P.P. and IYER, K.M. (1958): A chemical examination of *Emblica officinali*s, GAERTN. *Curr. Sci.* **3:** 266–267.

POLLAK, V.A. (1987): Linearity conciderations in quantitative thin-layer chromatography. *J. Chromatogr.* **393:** 143–153.

POOLE, S.K., KIRIDENA, W., MILLER, K.G. and POOLE, C.F. (1995): Planar chromatographic methods for determination of the quality of spices and flavors as exemplified by cinnamon and vanilla. *J. Planar Chromatogr.* **8:** 257–268.

POOLE, C.F. and POOLE S.K. (1994): Instrumental thin-layer chromatography. *Anal. Chem.* **66:** 27A–37A.

POWELL, W.S. (1985): Reversed-phase high-pressure liquid chromatography of arachidonic acid metabolites formed by cyclooxigenase and lipoxygenases. *Anal. Biochem.* **148:** 59–69.

QUADRY, S.M.J.S., BANGA, S.S. and ATAL, C.K. (1962): Pharmacognostic study of the fruits of *Emblica officinalis* GAERTN. *Ind. J. Pharm.* **24:** 2–6.

QUECKENBERG, O.R. and FRAM, A.W. (1993): Chromatographic and spectroscopic coupling: a powerful tool for the screening of wild Amaryllidaceae. *J. Planar Chromatogr.* **6:** 55–61.

RAM, S. and RAJA, T. (1978): Studies on naturally occuring gibberellins in aonla (*Emblica officinalis*) fruit. *New Phytol.* **81:** 513–519.

RAM, S. and RAO, T.R. (1976): Naturally occuring cytokinins in aonla (*Emblica officinalis*) fruit. *New Phytol.* **76:** 441–448.

RAUWALD, H.W., BREHM, O. and ODENTHAL, K.P. (1994): Screening of nine vasoactive medicinal plants for their possible calcium antagonistic activity. Strategy of selection and isolation for the active principles of *Olea europaea* and *Peucedanum ostruthium*. *Phytother. Res.* **8:** 135–140.

RENGER, B., JEHLE, H., FISCHER, M. and FUNK, W. (1995): Validation of analytical procedures in pharmaceutical analytical chemistry: HPTLC assay of theophylline in an effervescent tablet. *J. Planar Chromatogr.* **8:** 269–278.

REVUELTA, M-P., CANTABRANA, B. and HIDALGO, A. (1997): Depolarization dependent effect of flavonoids in rat uterine smooth muscle contraction elicited by $CaCl_2$. *Gen. Pharmacol.* **29:** 847–857.

RICE-EVANS, C.A. and PACKER L., Eds. (1998): Flavonoids in health and disease. Marcel Dekker Inc., New York.

ROSALES, C. and BROWN, E.J. (1992): Calcium channel blockers nifedipine and diltiatzem inhibit Ca^{2+} release from intracellular stores of neutrophils. *J. Biol. Chem. 267:* 1443–1448.

ROY, A.K., DHIR, H., SHARMA, A. (1991): Comparative efficacy of *Phyllanthus emblica* fruit extract and ascorbic acid in modifying hepatotoxic and renotoxic effects induced by metals *in vivo*. *Int. J. Crude Drug Res. Int. J. Pharmacognosy* (Netherlands). **29:** 117–126.

ROY, A.K., DHIR, H., SHARMA, A. (1992): Modification of metal-induced micronuclei formation in mouse bone marrow erythrocytes by *Phyllanthus* fruit extract and ascorbic acid. *Toxicol. Lett.* **62:** 9–17.

ROZYLO, J.K. and JANICKA, M. (1991): Thermodynamic description of liquid-solid chromatography process in the optimization of separation conditions of organic compound mixture. *J.Liq. Chromatogr.* **14:** 3197–3212.

ROZYLO, J.K. and JANICKA, M. (1996): Different planar techniques for prediction of solute retention in column liquid chromatography. J. *Planar Chromatogr.* **9:** 418–424.

SAITO, K., SANO, H., KAWAHARA, J. and YOKOHAMA, M. (1995): Calcium supplementation attenuates an enhanced platelet function in salt-loaded mildly hypertensive patients. *Hypertension* **26:** 156–163.

SAMMAN, S., LYONS WALL, P.M. and COOK, N.C. (1998): Flavonoids and coronary hearth disease: dietary perspectives, pp 469–481. In: Flavonoids in health and disease, Rice-Evans, C.A. and Packer, L. Eds. Marcel Dekker Inc., New York.

SANKARANARAYANAN, J. and JOLLY, C.I. (1993): Phytochemical, antibacterial and pharmacological investigations on *Momordica charantia* Linn., *Emblica officinalis* Gaertn and *Curcuma longa* Linn. *Indian J. Sci.* **1:** 6-13.

SENSCH, O.W., VIERLING, W., BRANDT, M. and REITER, M. (1993): Eugenol and caryophyllene oxide as calcium current inhibitors. *N.S. Arch. Pharmacol.* **347:** R78.

SHAH, F.H. and HAMID, A. (1968): Studies in amla fruit (*Emblica officinalis). Sci. Ind. (Karachi)* **6:** 50–56.

SHAHIDI, F., JANITHA, P.K. and WANASUNDARA, P.D. (1992): Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr.* **32:** 67–103.

SHERMA, J. (1994): Planar chromatography. *Anal. Chem.* **66:** 67R–83R.

SIRÉN, H., JUMPPANEN, J.H., MANNINEN, K. and RIEKKOLA, M-L. (1994): Introduction of migration indices for identification: chiral separation of some ß-blockers by using cyclodextrins in micellar electrokinetic capillary chromatography. *Electrophoresis* **15:** 779–784.

SMITH, W.L., MEADE, E.A. and DEWITT, D.L. (1994): Pharmacology of prostaglandin endoperoxide synthase isozymes-1 and -2. *Ann. NY Acad. Sci.* **714:** 136–142.

SMOLEN, J.E. (1992): Neutrophil signal transduction: calcium, kinases, and fusion. *J. Lab. Clin. Med.* **120:** 527–532.

SNYDER L.R. (1978): Classification of the solvent properties of common liquids. J. *Chromatogr. Sci.* **16:** 223–234.

SNYDERMANN, R. and UHING, R.J. (1992): Chemoattractants stimulus-response coupling, pp. 421–439. In: Inflammation: Basic principles and Clinical Correlates, Gallin, J.I. Goldstein, I.M. and Snydermann, R., Eds., Raven Press Ltd., New York.

SOMSEN, G.W., MORDEN, W. and WILSON, I.D. (1995): Planar chromatography coupled with spectroscopic techniques. *J. Chromatogr. A* **703:** 613–665.

SPEDDING, M. and CAVERO, I. (1984): "Calcium antagonists": A class of drugs with a bright future. Part II. Determination of basic pharmacological properties. *Life Sci.* **35:** 575–587.

SRIVASTAVA, S.K. and RANJAN, S. (1967): Physiological studies on plant tannins III. Variation of tannin compounds in the developing fruits of *Emblica officinalis* (GAERTN). *Flora Allg. Bot. Zeit (Jena)* **158:** 133–141.

STAHL, E. (1967): Dünnschichtchromatographie. Springer-Verlag, Berlin–Heidelberg–New York.

STAHLMANN, S., FREY, O. and KOVAR, K-A. (1998): Stability-indicating HPTLC-FTIR method for the quantitative analysis of fosfomycin-disodium in pharmaceutical preparations, pp. 158–168. In: Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, Visegrad, Hungary, 16–19.05.1998.

STICH, H.F. and ROSIN M.P. (1984): Naturally occuring phenolics as antimutagenic and anticarcinogenic agents. *Adv. Exp. Med. Biol.* **177:** 1–29.

STRASTERS, J.K. and KHALEDI, M.G. (1991): Migration behaviour of cationic solutes in micellar electrokinetic capillary chromatography. *Anal. Chem.* **63:** 2503–2508.

SUBRAMANIAN, S.S., NAGARAJAN, S. and SULOCHANA, N. (1971): Euphorbiaceae. Flavonoids of some Euphorbiaceous plants. *Phytochemistry* **10:** 2548–2549.

SUMMANEN, J., unpublished results.

SUMMANEN, J., HILTUNEN, R. and VUORELA, H. (1998): The choice of parameters in the optimization of automated multiple development. *J. Planar Chromatogr.* **11:** 16–24.

SUMMANEN, J., VUORELA, H., HILTUNEN, R., SIRÉN, H. and RIEKKOLA M-L. (1995): Determination of phenolic antioxidants by capillary electrophoresis with ultraviolet detection. *J. Chromatogr. Sci.* **33:** 704–711.

SUMMANEN, J., VUORELA, P. RAUHA, J-P., TAMMELA, P., MARJAMÄKI, K., PASTERNACK, M., TÖRNQUIST, K. and VUORELA H. (1999): Contrasting effects of simple aromatic compounds and flavonoids on calcium fluxes in clonal rat pituitary GH_4C_1 cells. *J. Pharmacol. Exp. Ther.* (submitted for publication).

SURESH, K. and VASUDEVAN, D.M. (1994): Augmentation of murine natural killer cell and antibody dependent cellular cytotoxicity activities of *Phyllanthus emblica*, a new immunomodulator. *J. Ethnopharmacol.* **44:** 55–60.

TAN, C.H., JAVORS, M.A., SELESHI, E., LOWRIMORE, P.A., and BOWDEN, C.L. (1990): Effects of lithium on platelet ionic intracellular calcium concentration in patients with bipolar (manic-depressive) disorder and healthy controls. *Life Sci.* **46:** 1175–1180.

TECKLENBURG, R.E., Jr., FRICKE, G.H. and NUROK, D. (1984): Overlapping resolution maps as an aid in parallel development of thin-layer chromatography. *J. Chromatgr.* **290:** 75–81.

TERABE, S., OTSUKA, K., ICHIKAWA, K., TSUCHIYA, A. and ANDO, T. (1984): Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* **56:** 111–113.

THAKUR, C.P. (1985): *Emblica officinalis* reduces serum, aortic and hepatic cholesterol in rabbits. *Experientia* **41:** 423–424.

THELEN, M., DEWALD, B. and BAGGIOLINI, M. (1993): Neutrophil signal transduction and activation of the respiratory burst. *Physiol. Rev.* **73:** 797–821.

THERESA, Y.M., RAJANDURAI, S., SASTRY, K.N.S. and NAYUDAMMA, Y. (1967): Studies on biosynthesis of tannins in indigenous plants XIII. Occurence of a new gallotannin amlaic acid in amla leaves (*Phyllanthus emblica*). *Leather Science* **14:** 16–17.

THERESA, Y.M., SASTRY, K.N.S. and NAYUDAMMA, Y. (1965): Studies on biosynthesis of tannins in indigenous plants XII. Occurence of different polyphenolics in amla (*Phyllanthus emblica* Linn). *Leather Science* **12:** 327–328.

THORAT, S.P., REGE, N.N., NAIK, A.S., THATTE, U.M., JOSHI, A., PANICKER, K.N.S., BAPAT, R.D. and DAHANUKAR, S.A. (1995): Emblica officinalis: a novel therapy for acute pancreatitis—an experimental study. *HPB Surgery* **9:** 25–30.

TIKKANEN, I. and JOHNSTON, C.I. (1997): Comparison of renin-angiotensin to calcium channel blockade in renal disease. *Kidney Int. Suppl.* **63:** S19–S22.

TITIEVSKY, A., TITIEVSKAYA, I., PASTERNACK, M., KAILA, K. and TÖRNQUIST, K. (1998): Sphingosine inhihibits voltage-operated calcium channels in GH_4C_1 cells. *J. Biol. Chem.* **273:** 242–247.

TOMÁS-BARBERÁN, F.A. (1995): Capillary electrophoresis: a new technique in the analysis of plant secondary metabolites. *Phytochem. Anal.* **6:** 177–192.

TOUCHSTONE, J.C. (1990): Currents in planar chromatography. Chem. Anal., **108** (Planar Chromatogr. Life Sci.): 1–5.

TOUCHSTONE, J.C. (1992): Practice of thin layer chromatography,. John Wiley & Sons Inc., New York–Chichester–Brisbane–Toronto–Singapore.

TROPEA, M.M., GUMMELT, D., HERZIG, M.S., LEEB-LUNDBERG, L.M.F. (1994): B_1 and B_2 kinin receptors on cultured rabbit superior mesenteric artery smooth muscle cells: receptor specific stimulation of inositol phosphate formation and arachidonic acid release by des-arg^9-bradykinin and bradykinin. *J. Pharmacol. Exp. Ther.* **264:** 930–937.

TÖRNQUIST, K. and TASHJIAN Jr., A.H. (1989): Dual actions of 1,25-dihydroxycholcalciferol on intracellular Ca^{2+} in GH_4C_1 cells: Evidence for effects on voltage-operated Ca^{2+} channels and Na^+/Ca^{2+} exchange. *Endocrinology* **124:** 2765–2775.

UNANDER, D.W., WEBSTER, G.L., and BLUMBERG, B.S. (1990): Records of usage or assays in *Phyllanthus* (Euphorbiaceae) I. Subgenera *Isocladus, Kirginelia, Cicca* and *Emblica*. *J. Ethnopharmacol.* **30:** 233–264.

UNANDER, D.W., WEBSTER, G.L., and BLUMBERG, B.S. (1991): Uses and bioassays in *Phyllanthus* (Euphorbiaceae): a compilation II. The subgenus *Phyllanthus*. *J. Ethnopharmacol.* **34:** 97–133.

UNANDER, D.W., WEBSTER, G.L., and BLUMBERG, B.S. (1992): Usage and bioassays in *Phyllanthus* (Euphorbiaceae): a compilation III. The subgenera *Eriococcus, Conami, Gomphidium, Botryanthus, Xylophylla* and *Phyllanthodendron*, a complete list of the species cited in the three-par series. *J. Ethnopharmacol.* **36:** 103–112.

UNANDER, D.W., WEBSTER, G.L., and BLUMBERG, B.S. (1995): Usage and bioassays in *Phyllanthus* (Euphorbiaceae) IV. Clustering of antiviral uses and other effects. *J. Ethnopharmacol.* **45:** 1–18.

VANE, J. (1994): Towards a better aspirin. *Nature* **367:** 215–216.

VANE, J.R. and BOTTING R.M. (1994): Regulatory mechanisms of the vascular endothelium: an update. *Pol. J. Pharmacol.* **46:** 499–521.

VEENSTRA, J. VAN DE POL, H., VAN DER TORRE, H., SCHAAFSMA, G. and OCKHUIZEN, T. (1988): Rapid and simple methods for the investigation of lipoxygenase pathways in human granulocytes. *J. Chromatogr.* **431:** 413–417.

VÉGH, Z., WISZKIDENSKY, A., NARANCSIK, A., FERENCZI-FODOR, K. (1998): Documentation of OPLC and TLC chromatograms by means of colour video technology and conventional colour photography, pp. 238–246. In: Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, Visegrad, Hungary, 16–19.05.1998.

VIEIRA, O., ESCARGUEIL-BLANC, I., MEILHAC, O., BASILE, J-P., LARANJINHA, J., ALMEIDA, L., SALVAYRE, R., and SALVAYRE-NÉGRE, A. (1998): Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidised LDL. *Br. J. Pharmacol.* **123:** 565–573.

VITEK, R.K. (1991): Photographic documentation of thin-layer chromatograms, pp. 211-248. In: Handbook of thin-layer chromatography, Sherma, J. and Fried, B., Eds. Marcel Dekker, Inc., New York–Basel–Hong Kong.

VOGEL H.G. and VOGEL W.H. (1997): Drug discovery and evaluation: pharmacological assays. Springer-Verlag, Berlin. Heidelberg.

VOVK, I., FRANKO, M., GIBKES, J., PROSEK, M. and BIBANIC, D. (1998): The effect of drying conditions on the in-depth distribution of compounds on TLC plates investigated by photoacoustic spectroscopy, pp. 402–410. In: Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, Visegrad, Hungary, 16–19.05.1998.

VOVK, I and PROSEK, M. (1997a): Quantitative evaluation of chromatograms from totally illuminated thin-layer chromatographic plates. *J. Chromatogr. A* **779:** 329–336.

VOVK, I. and PROSEK, M. (1997b): Reproducibility of densitometric and image analysing quantitative evaluation of thin-layer chromatograms. *J. Chromatogr. A* **768:** 329–333.

VUORELA, H. (1988): Chemical and biological study on coumarins in *Peucedanum palustre* roots, thesis, 64 p., University of Helsinki, J-Paino Ky, Helsinki.

VUORELA, H., DALLENBACH–TÖLKE, K., HILTUNEN, R. and STICHER, O. (1988): Separation of the main coumarins of Peucedanum palustre with various planar chromatographic methods. *J. Planar Chromatogr.* **1:** 123–127.

VUORELA, H., DALLENBACH–TÖLKE, K., NYIREDY,SZ., HILTUNEN, R. and STICHER, O. (1989): Optimization of the HPLC separation for the main furocoumarin isomers from *Peucedanum palustre*. *Planta Med.* **55:** 117–234.

VUORELA, H., VUORELA, P., TÖRNQUIST, K. and ALARANTA S. (1997): Calcium channel blocking activity: screening methods for plant derived compounds. *Phytomedicine* **2:** 167–181.

WAGNER, H. and DORSCH, W. (1992): Offenlegungscrift DE 41 06 036 A 1 (1992) and U.S. Patent 5260335 (1992).

WANG, Q-S., GAO, R-Y. and WANG H-Y. (1989): Computer-assisted transposition of conditions in high-performance thin-layer chromatography to high-performance liquid chromatography for the separation of pesticides. *Chromatogaphia* **28:** 285–288.

WANG, Q-S., YAN, B-W. and ZHAO J-H. (1998): Computer program of chromatography (II)-monofactor and difactor statistical optimum system in HPTLC, pp. 265-273. In: Proceedings of the 10th International Symposium on Instrumental Planar Chromatography, Visegrad, Hungary, 16–19.05.1998.

WATSON, M.W. and CARR, P.W. (1979): Simplex algorithm for the optimization of gradient elution high-performance liquid chromatography. *Anal. Chem.* **51:** 1835–1842.

WEINS, C. and HAUCK, H.H. (1996): Advances and developments in thin layer chromatography. *LC-GC Int.* **9:** 710–717.

WEITHMANN, K.U., JESKE, S. and SCHLOTTE, V. (1994): Effect of leflunomide on constitutive and inducible pathways of cellular eicosanoid generation. *Agents Actions* **41:** 164–170.

WILSON, I.D., SPRAUL, M. and HUMPFER, E. (1997): Thin-layer chromatography combined with high-resolution solid-state NMR for compound identification without substance elution: preliminary results. *J. Planar Chromatogr.* **10:** 217–219.

WYATT, D.M. (1981): Simultaneous analysis of BHA, TBHQ, BHT and propyl gallate by gas chromatography as extracted from refined vegetable oil. *J. Am. Oil Chem. Soc.* **58:** 917–920.

YADAV, S.K. (1987): Protection against radiation induced chromosome damage by *Emblica officinalis* fruit extract. *Caryologia* **49:** 261–265.

YAMAHARA, J., MIKI, S., MURAKAMI, H., SAWADA, T. and FUJIMURA, H. (1985): Screening test for calcium antagonists in natural products and the active principles of *Cnidii monnieri*. *Yakugaku Zasshi 105:* 449–458.

YANO, S., HORIUCHI, H., HORIE, S., AIMI, N., SAKAI, S.-I. and WATANABE, K. (1991): Ca^{2+} channel blocking effects of hirsutine, an indole alkaloid from *Uncaria* genus, in the isolated rat aorta. *Planta Med.* **57:** 403–405.

YRJÖNEN, T., SUMMANEN, J., REMES, S., VUORELA, H, VUORELA, P. and HILTUNEN, R.: Antibacterial activity of extracts and constituents of *Phyllanthus emblica* L. (Euphorbiaceae), unpublished results.

ZEE-CHENG, R.K.-Y. AND CHENG, C.C. (1986): Ellagic acid. *Drugs of the Future* **11(12):** 1029–1033.

ZOGG, G.C. (1989): Optimization and transfer of the mobile phase between various analytical and preparative liquid chromatographic methods, thesis 141 p., ETH Zürich, No. 8780, Switzerland.

ZOGG, G.C., NYIREDY, Sz. and STICHER, O. (1988): Comparison of fully off-line and fully on-line OPLC with HPLC illustrated by the separation of furocoumarin isomers. *J. Planar Chromatogr.* **1:** 351–354.